"Effects of Trichothecenes on Cardiac Cell Electrical Function"

FINAL REPORT

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16 December 1985

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

Fort Detrick, Frederick, Maryland 21701-5012

Grant Number DAMD17-83-G-9563

University of Alabama at Birmingham

Birmingham, Alabama 35294

A125 949

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188
Exp Date Jun 30, 1986

1a REPORT SECURITY CLASSIFICATION: Unclassified	1b RESTRICTIVE MARKINGS
2a SECURITY CLASSIFICATION AUTHORITY	3 DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b DECLASSIFICATION/DOWNGRADING SCHEDULE	
4 PERFORMING ORGANIZATION REPORT NUMBER(S)	5 MONITORING ORGANIZATION REPORT NUMBER(S)
6a NAME OF PERFORMING ORGANIZATION: University of Alabama Birmingham; 6b OFFICE SYMBOL (If applicable)	7a NAME OF MONITORING ORGANIZATION
6c ADDRESS (City, State, and ZIP Code): Birmingham, Alabama 35294	7b ADDRESS (City, State, and ZIP Code)
8a NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command; 8b OFFICE SYMBOL (If applicable)	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-83-G-9563
8c ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10 SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 62770A; PROJECT NO. 3M162. 770A871; TASK NO. AA; WORK UNIT ACCESSION NO 356

11 TITLE (Include Security Classification)

(U) Effects of Trichothecenes on Cardiac Cell Electrical Function

12 PERSONAL AUTHOR(S)

W. T. Woods, Jr.

13a TYPE OF REPORT	13b TIME COVERED	14 DATE OF REPORT (Year, Month, Day)	15 PAGE COUNT
Final Report	FROM 10/83 TO 9/85	16 December 1985	81

16 SUPPLEMENTARY NOTATION

17 COSATI CODES			18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	
06	13		
06	03		

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

Exposure to relatively small amounts of trichothecenes causes sudden death in humans and experimental animals. Prior to death, heart function becomes abnormal. Therefore, trichothecenes may have lethal effects on cardiac cells or on the nerves in the heart. This project determined how trichothecenes affect electrical activity in heart cells and how trichothecenes affect neural control of the circulation. Phase I addressed the effects of T-2 toxin and roridin-A (macrocyclic trichothecene) on heart cell electrophysiology in isolated, arterially perfused tissues from dog hearts. Cells in the sinus node pacemaker, atrial wall, atrioventricular node, atrioventricular bundle, false tendons, and ventricular wall were impaled with microelectrodes during arterial perfusion of each toxin to assess changes in rate of beating, conduction velocity, and action potential morphology. Effects of sympathetic and parasympathetic nerves during toxin perfusion were revealed by blocking their receptors with proranolol and atropine, respectively. In Phase II the same toxins were perfused while the heart remained in the

20 DISTRIBUTION/AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	
22a NAME OF RESPONSIBLE INDIVIDUAL: Mrs. Judy Pawlus; 22b TELEPHONE (Include Area Code): 301-663-7325; 22c OFFICE SYMBOL: SGRD-RMI-S	

DD FORM 1473, 84 MAR

83 APR edition may be used until exhausted.
All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE

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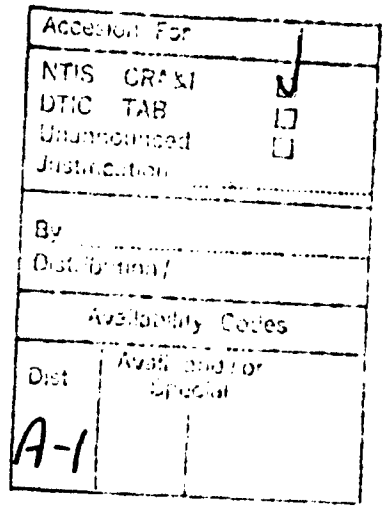
chest with its nerves and vessels intact and with unipolar electrodes attached. Responses to toxins were compared to the cellular electrophysiologic responses recorded in Phase I. Responses to sympathetic and parasympathetic nerve stimulation were assessed during toxin injection. In Phase III, the role of the heart in the cardiovascular system response to trichothecenes was assessed with the techniques used in Phase II along with arterial and venous pressure measurements. This study filled a conspicuous gap in our knowledge since there was virtually no information previously available about how trichothecenes affect the heart.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).



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(1) Statement of the problem under study.

Exposure to relatively small amounts of trichothecenes causes sudden death in humans and experimental animals. Prior to death, hea function becomes abnormal. Therefore, trichothecenes may have lethal effects on cardiac cells or on the nerves in the heart. This project determined how trichothecenes affect electrical activity in heart cells and how trichothecenes affect neural control of the circulation. Phase I addressed the effects of T-2 toxin and roridin-A (macrocyclic trichothecene) on heart cell electrophysiology in isolated, arterially perfused tissues from dog hearts. Cells in the sinus node pacemaker, atrial wall, atrioventricular node, atrioventricular bundle, false tendons, and ventricular wall were impaled with microelectrodes during arterial perfusion of each toxin to assess changes in rate of beating, conduction velocity, and action potential morphology. Effects of sympathetic and parasympathetic nerves during toxin perfusion were revealed by blocking their receptors with propranolol and atropine, respectively. In Phase II the same toxins were perfused while the heart remained in the chest with its nerves and vessels intact and with unipolar electrodes attached. Responses to toxins were compared to the cellular electrophysiologic responses recorded in Phase I. Responses to sympathetic and parasympathetic nerve stimulation were assessed during toxin injection. In Phase III, the role of

the heart in the cardiovascular system response to trichothecenes was assessed with the techniques used in Phase II along with arterial and venous pressure measurements. This study filled a conspicuous gap in our knowledge since there was virtually no information previously available about how trichothecenes affect the heart.

(2) Background and review of literature.

Chronic ingestion of certain toxins produced by Fusarium and Stachybotrys causes gastrointestinal lesions and bleeding (1,4,5,12,14,15,16,17,20,21,25). Protein synthesis is inhibited by these sesquiterpenoids which are known as trichothecenes (8). Some are carcinogenic (22,23). Acute exposure to high doses of trichothecenes caused sudden death in horses, cattle, sheep, swine, and poultry (8). The primary causes of death were unknown, but the cardiovascular system always appeared to be involved (8,9,12,21,22,23) and there has been frequent evidence of neurologic dysfunction (1,4,8,9,12,15,16,21,22,23).

Literature concerning the cardiovascular system in acute trichothecene toxicity is scarce. A few reports of disturbances in cardiac function have appeared (8,9,12,21, 22,23). These include rapid heart rate, slow heart rate and irregular heart rate; the electrocardiogram becomes abnormal (see reference 8 for review). The output of the heart is the product of the heart rate and the stroke volume (amount of blood pumped in each heartbeat). When the rate of

beating becomes too rapid, the interval for filling the heart becomes too short, and therefore, stroke volume falls. When the rate of beating becomes too slow, the output becomes too low to sustain all organs. Irregularity of heart rate (tachyarrhythmias or bradyarrhythmias) can cause either problem and is a type of cardiac electrical instability that can have fatal consequences.

Other disturbances in cardiac function during acute trichothecene toxicity may not have been documented in previous studies simply because they are less obvious. For example, abnormal conduction of the impulse through the heart could alter the sequence of activation of the heart muscle cells, reducing cardiac pumping efficiency. A complicating factor could be depression of contractile performance in heart cells. This lowers cardiac output by diminishing the heart's ability to eject blood. Each of these problems and those documented previously could result from direct effects of trichothecenes on individual heart cells. They could also be secondary to trichothecene action in the nervous system, since sympathetic nerves and parasympathetic nerves are abundant in the mammalian heart.

Previous observations in animals suffering from acute trichothecene toxicity have implicated disturbances in the nervous system (1,4,8,9,12,16,21,22,23), since the functions of innervated organs (skeletal muscle, lungs, heart, etc.) became altered (8). Central nerves may also be involved since motor disturbances, vomiting, psychoses, impaired

reflexes, seizures, and visual disturbances, were observed during trichothecene toxicity (5,8,21). The toxins may, therefore, affect nerve cell membranes or synapses with effector cells (neuromuscular and myoneural junctions) or both.

Past studies of acute trichothecene toxicity have produced mainly lists of signs and symptoms (8). By combining isolated heart studies with whole animal studies, this project determined mechanisms for trichothecene toxic effects. These studies demonstrated unequivocal reversible effects of certain mycotoxins on heart cell electrical activity.

Preliminary studies were carried out prior to beginning this project.

Trichothecene toxins were obtained from Sigma Chemical Co. (St. Louis). One mg. of each toxin was added to 0.1 mL of acetone and then to 9.9 mL of physiologic solution (see Experimental Methods); this became the stock solution [100 parts per million (ppm)]. The concentration tested upon heart cells was 1 ppm. Sprague-Dawley rats (from Charles River) were anesthetized and their hearts were excised and perfused (2.0 mL./min.) through the coronary arteries with physiologic solution at 37°C.

One of the most potent naturally-occurring trichothecene toxins is known as T-2. Four closely related derivatives of T-2 toxin (T-2 Triol, T-2 Tetraol, HT-2, and verrucarol) were tested by perfusion into 3 isolated

spontaneously beating rat hearts. Results are given in Table 1. Heart rate began to increase within the first 3 minutes. By 30 min., however, heart rate had fallen and continued to fall unless the toxin was removed. Then the rate became irregular with extrasystoles and re-entrant tachycardias appearing in atria and ventricles. Action potential duration was reduced suggesting that shorter refractory periods contributed to the arrhythmias. During this period action potentials in atria and ventricles acquired diminished upstroke velocities, although, the resting transmembrane potentials were unchanged. After 2 hrs. of toxin perfusion, the heart cells became quiescent and inexcitable. However, when the perfusion was limited to 30 min. effects of each toxin were completely reversible. Recovery to the control rate (222 beats per min.) required 12 ± 2 min. Action potentials recovered to normal even before the rate returned to normal.

Responses of each isolated perfused rat heart to these T-2 trichothecenes were reproducible and virtually identical. The effects of each toxin tested were also virtually identical. Action potentials of rat atrial and ventricular muscle cells acquired shorter durations and uniformly diminshed maximum upstroke velocities, suggesting impending conduction disturbances. This was supported by the observations that both the rapid (early) and the slow (later) heart rates were often irregular (dysrhythmic). Thus, several factors combined to disturb the activity of the rat heart.

Table 1. Effects of T-2 Trichothecenes on Atrial Action Potentials of 3 Rat Hearts.

	Rate at 3 min. (beats/min.)	Rate at 30 min. (beats/min.)	APD at 30 min. (msec.)	Rhythm at 30 min.	Rate, excitability at 2 hr.
Control	222 ± 60	222 ± 60	44 ± 4	Normal sinus rhythm with normal A-V condution	Normal sinus rate; normal action potentials
Verrucarol, T-2 Triol, T-2 Tetraol, or HT-2	256 ± 100	142 ± 50	28 ± 15	Atrial and Ventricular Tachyarrhythmias	Quiescent, Inexcitable

The rat heart was selected for these pilot studies because of its low cost. This laboratory prefers to use the canine heart as a model of the human heart since 1) the canine heart is electrophysiologically and anatomically very similar to the human heart, and 2) there is already abundant information on electrophysiology, anatomy, fine structure, biochemistry, and pharmacology of the canine heart.

(3) Rationale and hypotheses tested.

1) Trichothecenes depress cardiac function by inhibiting automaticity and impulse conduction.

Abnormal rates of beating were observed in pilot studies of trichothecene effects. Also, in the pilot studies cardiac action potential maximum upstroke velocity became reduced and this is often associated with slowing of the impulse conduction velocity in heart cells.

2) Trichothecenes disturb cardiac electrical activity by releasing autonomic neurotransmitters.

The increased heart rate followed by a decreased heart rate that was observed in the pilot studies of T-2 trichothecenes could result from release of norepinephrine (increase in rate) and acetylcholine (decrease in rate) from neural elements in the rat heart. This pattern was observed when tetraethylammonium or 4-aminopyridine was perfused in the isolated dog heart or when cardiac nerves were stimulated in the isolated heart (reference 32 and Figure 1). This hypothesis was tested in isolated canine heart tissue first; parallel studies evaluating the same

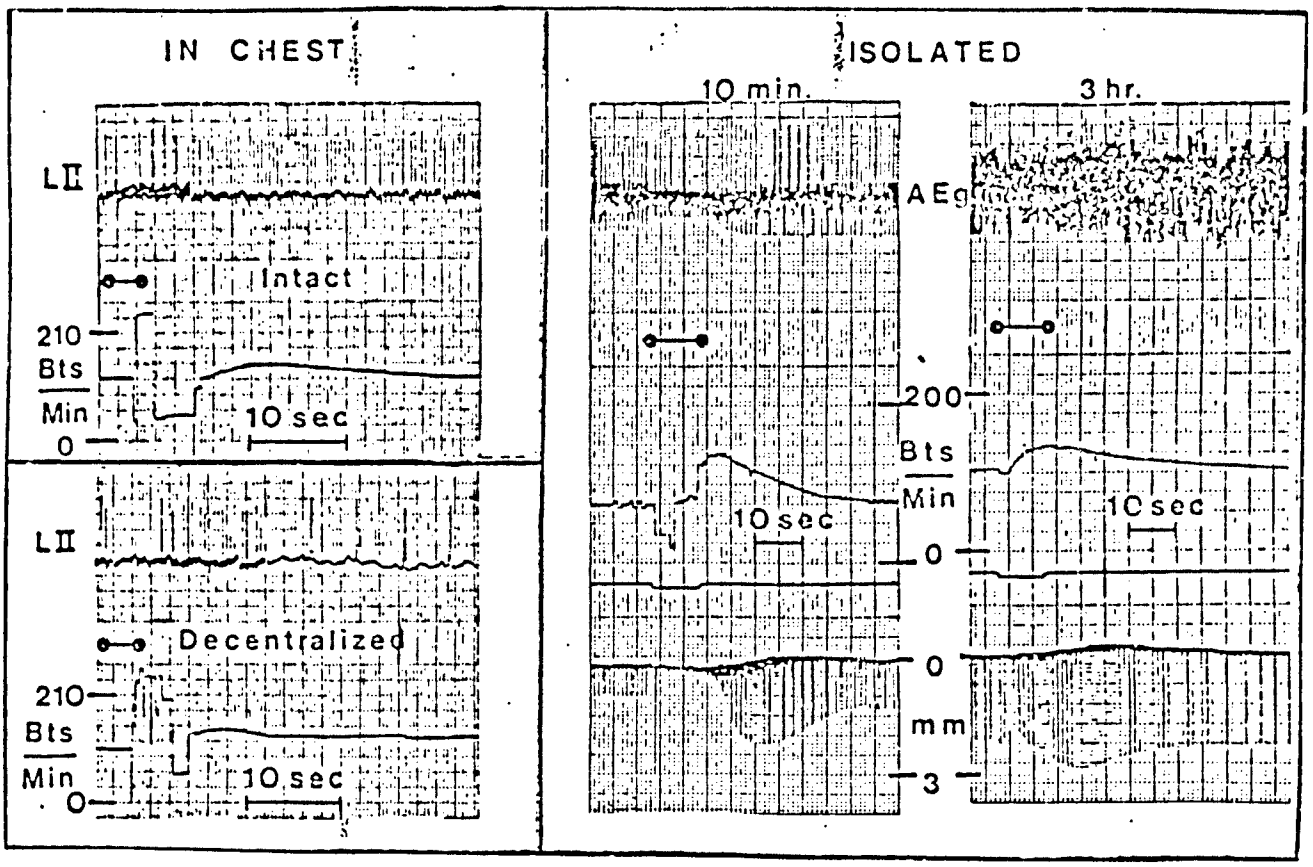


Figure 1. A cardiac nerve branch from the vagosympathetic trunk was isolated in the anesthetized open-chest dog while a Lead II electrocardiogram and tachogram were recorded. The nerve was stimulated (•——•) before it was severed (upper left), after cutting nerve away from vagus (lower left) and after transfer of the heart to the perfusion chamber (right). Note that each stimulation produced a decrease followed by an increase in heart rate. An atrial electrogram (AEg) substitutes for the electrocardiogram in the isolated heart.

responses in intact animals exposed to trichothecenes were carried out.

Sudden death can occur after acute exposure to trichothecenes. Since irregular heart rates and changes in the electrocardiogram have been observed in trichothecene-toxic animals, dysfunction of the heart is suggested as the cause of sudden death. However, no information is available about how trichothecenes affect the heart and the parts of the nervous system that control the heart. This study provided this information by examining the effects of trichothecenes upon the dog heart at the level of the single cell, the intact organ, and the cardiovascular system.

Effects of trichothecenes at the cellular level. The first studies perfomed (Phase I) established the effects of the toxins upon action potentials recorded in cardiac cells [pacemaker, atrial muscle, slowly conducting atrioventricular (AV) node, false tendon, and ventricular muscle cells] and upon the nerves in the heart (sympathetic and parasympathetic). These were performed with intracellular microelectrodes in isolated, arterially perfused segments of the heart as described in the Experimental Methods section and in previous publications from this laboratory (13,14,24,26-32).

Effects of trichothecenes at the organ level. As the cellular responses to the toxins were established, the whole heart responses to the same doses of toxins (Phase II) were

assessed. For these experiments the hearts were left intact in the dogs and selected arteries were perfused with the appropriate toxins. For example, the pacemaker's individual response was assessed by selectively injecting a toxin into the sinus node artery while the rest of the heart remained perfused by blood in the coronary arteries. The autonomic nerves that control heart activity were externally stimulated to test for any changes in neural responsiveness.

Effects of trichothecenes at the system level. The cellular and organ responses to these toxins were also analyzed during intravenous injection of the same toxins at the same concentrations. This was carried out in the anesthetized dog while cardiovascular responses were monitored (Phase III).

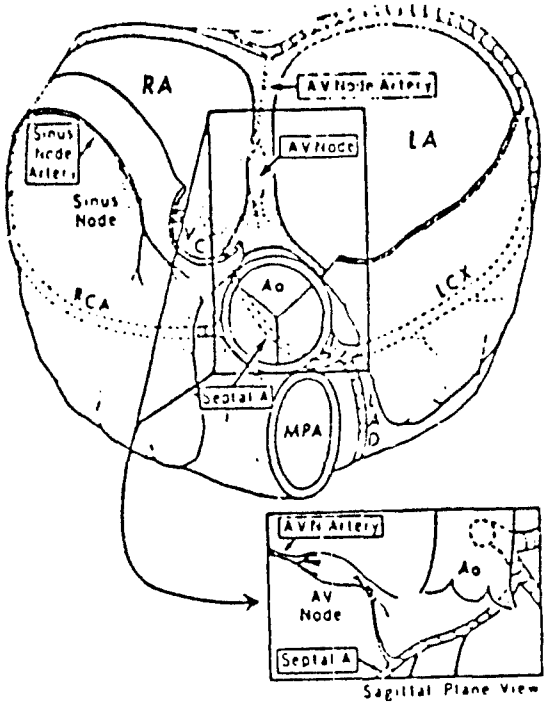
(4) Experimental methods.

Ninety-six mongrel dogs per year (12 ± 10 months old; half male, half female) were anesthetized with intravenous pentobarbital sodium (30 mg/kg). Their hearts were excised through a thoracotomy as previously described (29). A catheter was inserted into the proximal right coronary artery to perfuse its sinus node branch, into the distal left circumflex artery to perfuse its AV node branch, and into the septal branch of the left anterior descending coronary artery. These three arteries provide all the primary blood supply to the canine conduction system (see Figure 2) and are relatively constant in their origin and distribution (28). All visible branches of the coronary

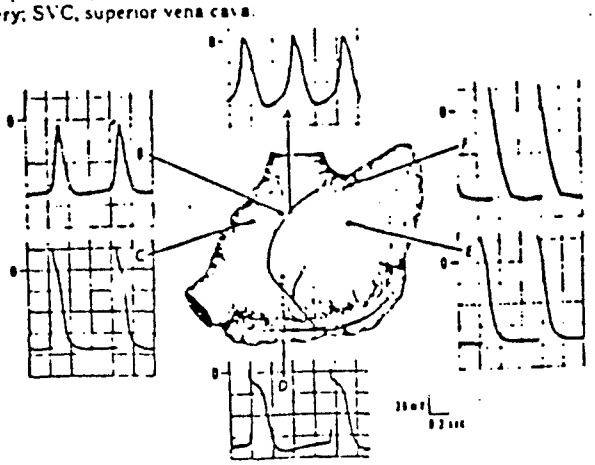
arteries that supply areas other than the sinus node or the AV junction were ligated with sutures. Both the continuing perfusion of the three cannulated arteries and the ligation of all other branches which may divert or leak the arterial flow are essential to the viability and continued normal performance of the structures under study. The perfusing solution contained (in millimoles per liter), Na^+ (145), K^+ (4.20), Ca^{++} (1.27), Mg^{++} (0.85), Cl^- (124), $SO_4^{=}$ (1.00), $H_2PO_4^-$ (2.40), HCO_3^- (25.0), and dextrose (5.6). The perfusate was pumped at a constant flow of 3 to 4 mL./min. into each catheter (9-12 mL./min. total). PO_2 exceeded 500 mm. Hg, pH was 7.4, and myocardial temperature was maintained at 36 ± 1°C. The arterial perfusate collected in the tissue chamber to submerge the entire preparation.

Once perfusion of all three cannulated arteris is established, the well-perfused regions become pale in comparison to any underperfused regions. After 15 min. of perfusion the well-demarcated underperfused regions were excised.

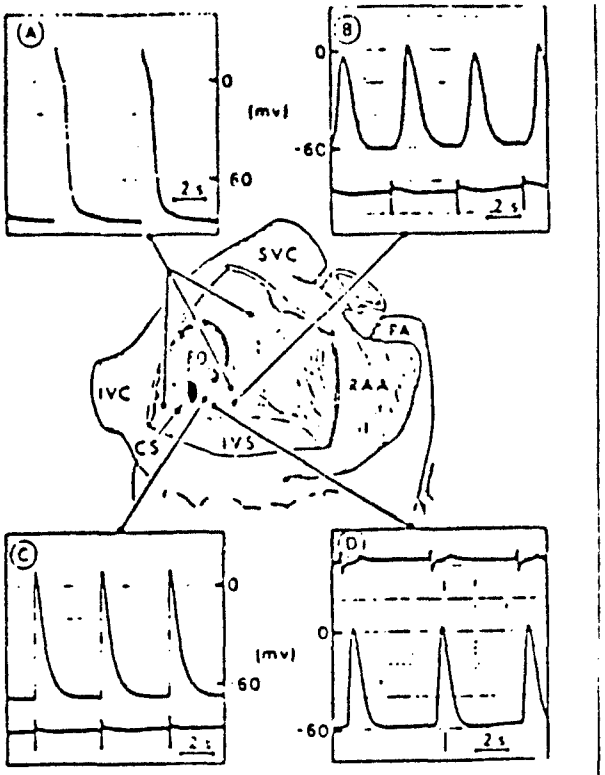
The final preparation for study included the right atrium, all of the interatrial septum, and the upper third of the interventricular septum intact; false tendons were included from the same heart. A cut was made in the right atrial free wall along the margin of the right atrial appendage from the AV sulcus to and through the stump of the superior vena cava. The lateral portion of the right atrial appendage then became a flap which was retracted to expose



Drawing shows in *dashed* and *solid outlines* the course of the 3 major arteries important for sinus node and AV node function in canine heart. Sinus node, AV node, and septal arteries were separately and simultaneously perfused in this study. Sinus node area is *stippled*. AV node is shown in side view with its dual blood supply. Ao, aorta; AVN, atrioventricular node; LA, left atrium; LCX, left circumflex artery; LAD, left anterior descending artery; MPA, main pulmonary artery; RA, right atrium; RCA, right coronary artery; septal A, septal artery; SVC, superior vena cava.



These transmembrane voltage recordings, photographed as they appeared on the oscilloscope graticule, were obtained during spontaneous sinus node activity. Lettered lines and dots designate the sites of impalements in the preparation drawn in the center. A, central sinus node. B, margin of sinus node. C, sinus intercavarum.



Action potentials typical of the ones recorded from endocardial surface of each isolated perfused canine atrium (reference locations drawn in center of Fig.). *Type A* was recorded in almost all sites containing working atrial muscle cells. In contrast, *type B* was typical of distal AV node region. *Types C* and *D* were recorded, respectively, with superficial and deeper AV node cell impalements. Bipolar surface electrograms in *B*, *C*, and *D* were recorded from atrial muscle adjacent to sinus node. Ao, aorta; CS, coronary sinus; FO, fossa ovalis; IVC, inferior vena cava; IVS, interventricular septum; PA, pulmonary artery; RAA, right atrial appendage; SVC, superior vena cava.

Figure 2. Upper left: Coronary arteries of the canine cardiac conduction system are shown. These would be perfused in the proposed study. Upper right: Typical action potentials from the canine endocardial surface and AV node. Lower: Typical action potentials from the canine epicardial surface and sinus node.

all of the right artial endocardial surface. The coronary sinus ostium and the fossa ovalis of the interatrial septum were centrally located in this exposed area. The tissues were held in position by pins stuck through their margins into an underlying plate of wax. All arteries transected by the final trimming procedure were ligated. Unless these leaks are tied, the preparation will not function satisfactorily. Upon completion of the preparation, a steady sinus rhythm produced visible contractions (29) at 115 ± 12 beats/min. Sinus rhythm and AV conduction, myocardial contractions, and transmembrane action potentials all remained stable for at least 5 hours.

A wax-bottomed plexiglass chamber (3-mL volume) was constructed (14,27) for superfusion of false tendons with a solution pumped at a rate of 25 mL./min. This size chamber is suitable for housing right ventricular false tendons that are quite variable in length and branching pattern. False tendons were obtained from canine hearts following the pentobarbital anesthesia and procedures described above. When false tendons were affixed to the wax floor of the perfusion chamber, the cells of this tissue were readily accessible to impalement with the same microelectrode arrangement used for impalement of atrial cells (described below). Cells within the false tendons were stimulated with a Grass S4 instrument in combination with an SIU5 isolation unit and a CCU 1A constant current unit. Rectangular stimulus pulses (2-msec duration) from two silver wires 2 mm

apart were adjusted so as to be as close to threshold as possible.

Autonomic nervous system drugs. These studies required perfusion of some drugs through the coronary arteries (30). Each was in the form of a powder dissolved in the normal perfusate. For beta-adrenergic receptor blockade, DL-propranolol hydrochloride (10 micrograms/mL, 2 mL) powder was dissolved in the normal perfusate. Atropine sulfate (10 micrograms/mL., 2 mL.) powder was added to the perfusate to produce acetylcholine receptor (muscarinic) blockade.

A possibility that had to be dealt with in these studies was that slower or faster heart rates per se may change electrical properties. It was important to consider that faster rates of pacemaker cell firing (produced for example by norepinephrine released by nerve endings in the sinus node) might have an effect on electrical properties. This, in control atria, the cells were paced by a surface bipolar electrode at rates corresponding to the rates observed during exposure to toxins. They were likewise paced at control rates during exposure to toxins. This revealed rate-related changes in action potentials.

Electrophysiological Techniques. Conventional micro-electrode techniques were used to record transmembrane potentials. Pyrex glass tubing (1 mm outside diameter) was heated and drawn to a tip having an outside diameter of less than 0.5 microns; these electrodes yield a direct current (dc) resistance of 1-50 megohms and tip potential less than

5 mV after being filled with 2.5 m KCl. The electrodes were suspended on the end of a spiraled silver wire (24-guage) which established the contact between the microelectrode and a microelectrode preamplifier. This method of mounting the electrode provided rigidity sufficient to pierce the epicardium and enough flexibility to accommodate tissue movement. When only one cell was impaled, a W-P Instruments M701 preamplifier was used. To record from two cells simultaneously, we used a high impedance preamplifier with capacity neutralization which can accept up to four microelectrode inputs. Its output was multiplexed for four-trace display into a single channel of a two-channel vertical amplifier (Hewlett-Packard 1806A). The second channel was used to display the epicardial surface potential recorded between two bare silver wires which were 2.5 mm in length and 1 mm apart. The bathing solution contacted a silver wire (30 gauge) coil which was grounded and served as the reference for all potential measurements. A rectangular calibration pulse of +50 mV applied between the coil and ground was used for capacity neutralization.

Because the excised, perfused right atrium contracts vigorously, some recordings of transmembrane potential became distorted by mechanical displacement of the microelectrode. The objective of this study, however, was to obtain action potentials from cells under conditions as similar as possible to those of a normal heart in situ. Recordings were considered acceptable only when the

following criteria were met: (1) at least three consecutive action potentials from a single cell were identical; and (2) after withdrawal from the cell the microelectrode tip resistance and potential were the same as before impalement. A storage oscilloscope (Hewlett-Packard 181A) was used to display transmembrane voltages and retain this display for photography with a Polaroid camera (Hewlett-Packard 197A).

For this study the glass capillary tips were honed by immersion in a 2.5 molar KCl-silicon carbide abrasive solution. The electrode d.c. resistance was thereby reduced to 10 ± 5 megohms. Single cell impalements were stable and deeper cell layers could be reached by the bevelled impaling tip. A silver unipolar electrode (2 mm. interpolar distance) was applied to the endocardial surface and provided a consistent timing reference for microelectrode recordings. When recording from cells with action potentials having a rapid upstroke, maximum upstroke velocity was monitored via the electronically differentiated signal and displayed simultaneously with the action potential trace. Upstroke velocity of sinus node action potentials was measured either directly from the tracings or automatically via a microprocessor.

To estimate the degree of coupling between heart cells, the technique descibed by Bonke (2,3) was employed. Microelectrodes were placed at 0.1, 0.5, 1.0 and 2.0 mm from the point source of current. Action potentials at the different points within the sinus node or atrial working

muscle were recorded within 2 min. to measure conduction times (intranodal or sinoatrial) and the apparent space constants. After 1 min, this was repeated. Thus, commencing at 6 min. prior to perfusing any test substances, conduction times and apparent space constants were measured at 3 min. intervals. This continued until complete recovery or until a new steady state (15 min.) was achieved.

Propagation from cell to cell is not linear. Therefore, use of single cell activation times to estimate conduction velocity between two points always under estimates the actual distance traveled by an impulse. When two cells are close together (within 30 microns) and oriented in a line parallel to their long axes, relative conduction velocities can be estimated by comparing activation times. In this study action potentials were recorded to calculate intervals between activation times of cells between 200 and 300 microns apart. Microelectrode tip positions were resolved within a 10-micron accuracy.

Research Protocols and Anticipated Chronology. Effects of naturally occurring trichothecenes and the macrocyclic series (23) were evaluated in the canine heart. The project was conducted in 3 phases that operated in parallel:

Phase I - cellular effects.

Phase II - cardiac effects.

Phase III - cardiovascular system effects.

Phase I required the entire 24 months. In this phase of the project, the effects of each toxin upon action

potentials recorded in each different kind of heart cell were determined. This included all parts of the dog heart that have been characterized anatomically and electrophysiologically in this laboratory:

1. sinus node pacemaker cells (26,27,29,30,31)
2. sinus node latent pacemaker cells (29)
3. atrial working cells (trabeculae and free wall) (14,26,28,29)
4. proximal atricventricular cells (28)
5. distal atrioventricular cells (28)
6. atrioventricular bundle cells (28)
7. false tendons (13,26,27)
8. ventricular working cells (papillary muscle and wall) (24,28)

Effects upon each type of cell required individual study and the large number of cell types accounts for the length of time required by Phase I.

Phase II involved perfusion of each toxin into selected coronary arteries of the in situ (anesthetized) dog heart. The same arteries perfused in the isolated hearts of the Phase I study were perfused in the Phase II study (sinus none, AV node, and septal arteries); the only difference was that the heart remained in the chest with its normal innervation and vascular connections. Resonses to T-2, diacetoxyscirpenol, verrucarin-A, roridin-A were compared to the Phase I responses. Since the whole heart rather than its minute subdivisions was studied in Phase II, it was

completed in fewer actual experiments.

Phase III was undertaken only after the cardiac response was understood. The aim of Phase III was to determine the heart's role in the overall cardiovascular system response to T-2, diacetoxyscirpenol, verrucarin-A, and roridin-A. This required cardiac and hemodynamic monitoring during intravenous infusion of selected quantities of the trichothecenes. The cardiovascular responses of intact, anesthetized dogs to each of the toxins were evaluated throughout the 2 year project.

Biohazard Safety Considerations. Cardiac tissue was obtained from dogs as described above; it was arterially perfused or suffused in vitro. The T-2 diacetoxyscirpenol, verrucarin-A, and roridin-A toxins (obtained from Sigma Chemical Co., St. Louis, Mo. or Makor Chemicals, Ltd., Jerusalem, Israel) were dissolved in 0.1 mL acetone or diethylether (20) and transferred to 9.9 mL perfusate per mg toxin. By adjusting the amounts of diluents, different concentrations of the toxins were available for perfusion. Hence, dose/response relationships were determined. All toxins in powder form were handled only under fume hoods. Disposable gloves, disposable nose/mount masks, and goggles were worn by personnel handling toxic powders and solutions. After use toxin-containing solutions were treated with hypochlorite (1.5%) for 30 min. to inactivate the toxins. Then the solutions were diluted 100-fold with tap water before disposal.

Schematic for 3-Phase Parallel Studies of Acute Trichothecene Toxicity in the Dog.

Phase I	Heart Cells Cardiac Nerves
Phase II	Cardiac Function Sympathetic Input Parasympathetic Input
Phase III	Cardiovascular System Autonomic Nervous System

Phase I. Electrophysiologic measurements that were made in isolated dog hearts include sinus rate, impulse conduction velocity, diastolic transmembrane potential, maximum upstroke velocity, overshoot, action potential duration at 20%, 50% and 80% repolarization (26). Use of microelectrodes established the occurrence of pacemaker shifts and emergence of atrioventricular junction pacemakers (28).

Changes in the above measurements can occur when sympathetic or parasympathetic nerve endings become activated. Therefore, each toxin was perfused before and after blockade of parasympathetic receptors by atropine (5 micrograms/mL) and beta-adrenergic receptors by propranolol (10 micrograms/mL) (30). This revealed components of the cellular electrophysiologic response of the heart to the toxins that were secondary to a sympathetic or parasympathetic nerve effect.

Each toxin was perfused for 45 minutes or until the response to it was stable for 15 min. Then 30 minutes or

more were allowed for recovery. When a new steady rate was achieved, another concentration of the same toxin was tested. Different concentrations (0.1, 1.0, 10, and 100 parts per million) were randomized and the investigator did not know which concentration was being perfused.

Because there were eight different cell types to be studied in these dog hearts. Phase I was subdivided into A) Effects of trichothecenes on sinus node pacemakers and atrial free wall working muscle cells; B) Effects of trichothecenes on atrial trabeculae, atrioventricular node, and atrioventricular bundle cells, and C) Effects of trichothecenes on false tendon, ventricular muscle, and papillary muscle cells. This allotted 8 months to each subdivision, and therefore, approximately 2 months for each toxin in each specific cardiac region.

Phase II. For this phase of the study, dogs were anesthetized with intravenous pentobarbital (30 mg./kg). They were intubated and placed on a respirator when the chest was opened. Sinus node, atrioventricular node, and septal arteries were catheterized so that a bolus of toxin could be injected into any specific region of the heart that was studied in Phase I.

Rich collateral blood flow sustains the perfused tissues and this was interrupted only during the 2 sec. injection of toxins (or their diluents) by hand-held syringe (11). Responses were compared to those observed with microelectrodes. Cardiac responses to stellate and vagus

stimulation (separately) were evaluated before and after delivery of the toxins. Likewise, effects of each toxin were tested during sustained stellate and vagal stimulation.

Although single cell responses cannot be readily recorded in these hearts, multicellular responses can be recorded by unipolar electrodes which demonstrate changes in conduction velocity and dysrhythmias. These electrodes were placed on the right atrium (near the sinus node), the right atrial appendage, and the left ventricle. Strain gauge arches were also attached to the heart to detect changes in force of contraction. As explained for isolated tissues, pacing was performed to account for rate-related changes in cardiac activity. As in Phase I, propranolol and atropine were injected to reveal and eliminate any effects that were neurally mediated. Because injection was local and brief, dose-response relationships could be relatively quickly elucidated. If recovery from a toxin was complete, another one could be tested in the same preparation.

Phase III. Preparation of the 48 animals was similar to Phase II except that all coronary arteries were left intact and hemodynamic monitoring was carried out. The cardiac electrodes and strain gauges were in place as in Phase II. Arterial pressure (femoral artery) and venous pressure (femoral vein) were measured simultaneously.

The major change in toxin delivery was that all tissues in the body were exposed, because the injection was intravenous. If a toxin had important vascular effects,

there were changes in arterial or venous pressure. Blockade of sympathetic or parasympathetic nerves with propranolol or atropine during trichothecene toxicity revealed the importance of any neural responses to the toxins.

Statistical Analysis. Toxin-induced changes in action potential morphology were assessed by unpaired data analysis with the Student T-test. In whole animal studies each dog served as its own control in most experiments. Thus, such experiments were analysed with paired data in the Student T-test. Differences were considered significant when the value of P was less than 0.05.

(5) Results

The overall research program was divided into 6 parts, each listed as a separate project in its own right. Contents of this results section are listed below.

PROJECT I. EFFECTS OF INTRAVENOUS T-2 AND RORIDIN A ON THE CANINE CARDIOVASCULAR SYSTEM.

Animals weighing 20 ± 5 kg. were anesthetized with intravenous pentobarbital (30 mg/kg). T-2 toxin or roridin-A (0.1, 1.0 and 3.0 mg/kg) were injected in one intravenous bolus of dimethyl sulfoxide (DMSO). Each injection was preceded by an equivalent volume of toxin-free DMSO to serve as a control for effects of DMSO per se. In 5 experiments certain responses to these toxins were immediate, but some required up to 2 hr. to develop. There was always a transient fall in arterial pressure and increase in heart rate. When this injection included T-2 toxin, there was after 5 min. a progressive increase in heart rate that reached a stable peak after 60 ± 15 min. (Figure 3B). In 4 separate experiments, for example, the increase was 145 ± 6 to 195 ± beats per min. (sinus tachycardia). During the period of increasing heart rate, arterial pressure was not significantly lower. This suggests that the elevated heart rate might not be a reflex-mediated response (to hypotension, for example). However, experiments were performed to test the role of norepinephrine which is the main sympathetic neurotransmitter in the mammalian heart. Propranolol (250 micrograms/kg.) was injected intravenously during T-2-induced tachycardia to block the beta-adrenergic receptor activated by norepinephrine (Figure 3C). In 3 experiments, this

lowered heart rate but only eliminated 1/2 of the T-2 induced increment in heart rate. Therefore, the data suggest that effects of T-2 on heart rate are mediated by neural release of norepinephrine as well as a direct effect on pacemaker cells.

The same number of experiments were performed in the same way to assess the cardiovascular effects of roridin-A. Responses were identical to those observed after intravenous T-2 except that 75 ± 30 min. after roridin-A the heart rate suddenly fell to a level suggesting sinus arrest or sino-atrial block of conduction (Figure 4). Electrocardiograms suggested that sinus arrest with emergence of a substitute pacemaker had taken place. Another marked response was the increased T-wave amplitude (Figure 5).

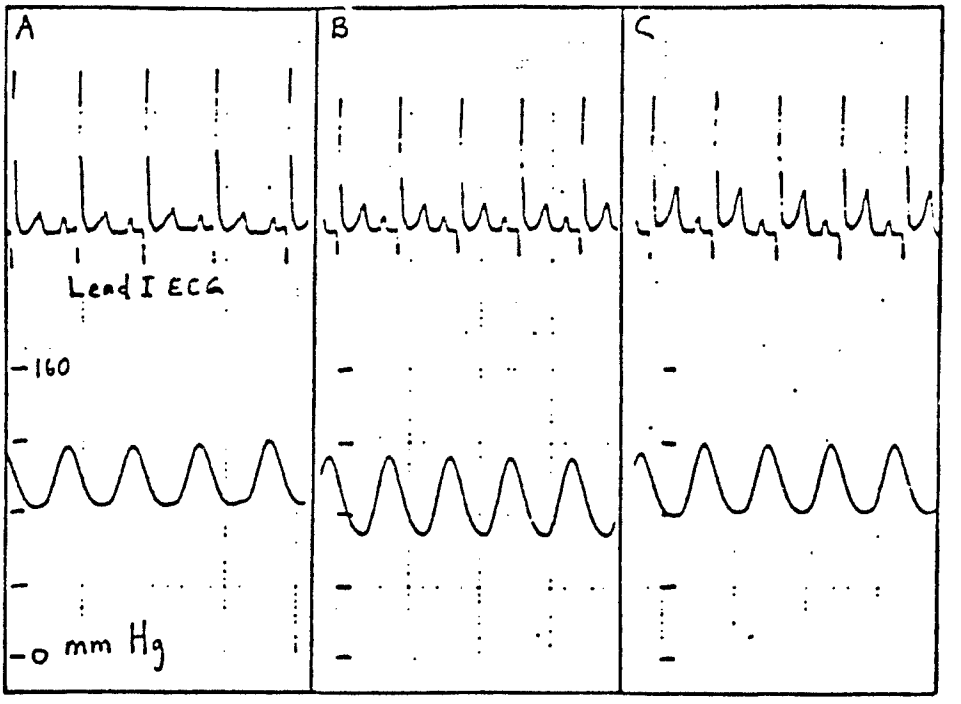


Figure 3. These panels show a lead 1 ECG (upper) and arterial pressure (lower) in an anesthetized animal before intravenous T-2 (1.5 mg./kg., panel A), 2 hours after T-2 (panel B), and 1 hour later following injection of propranolol (5 mg.) (panel C) 1.0 cm. = 0.400 sec. Note especially that only part of the T-2-induced tachycardia (150 to 176 bpm) was blocked by propranol (165 bpm). There was a time-dependent increase in T-wave amplitude suggesting hyperkalemia, but P-waves remained prominent suggesting the opposite.

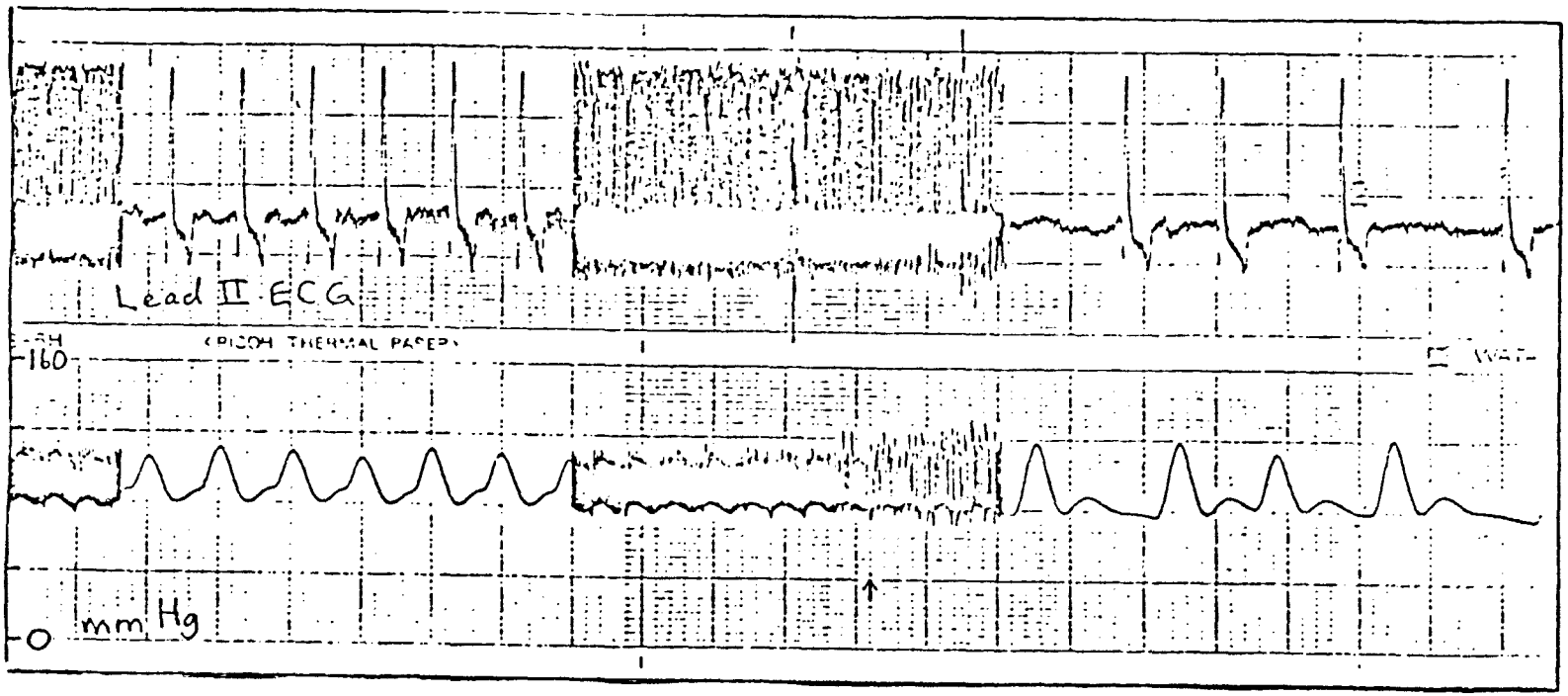


Figure 4. This continuous record shows the transition from normal impulse conduction to second degree atrioventricular block observed after 1 hour of intravenous roridin-A 2.0 mg./kg. The lead II ECG (upper trace) and arterial pressure truce (lower trace) show the irregular rate associated with this arrhythmia which began approximately at the arrow. Fast speed 25 mm./sec. and slow speed = 25 mm./min.

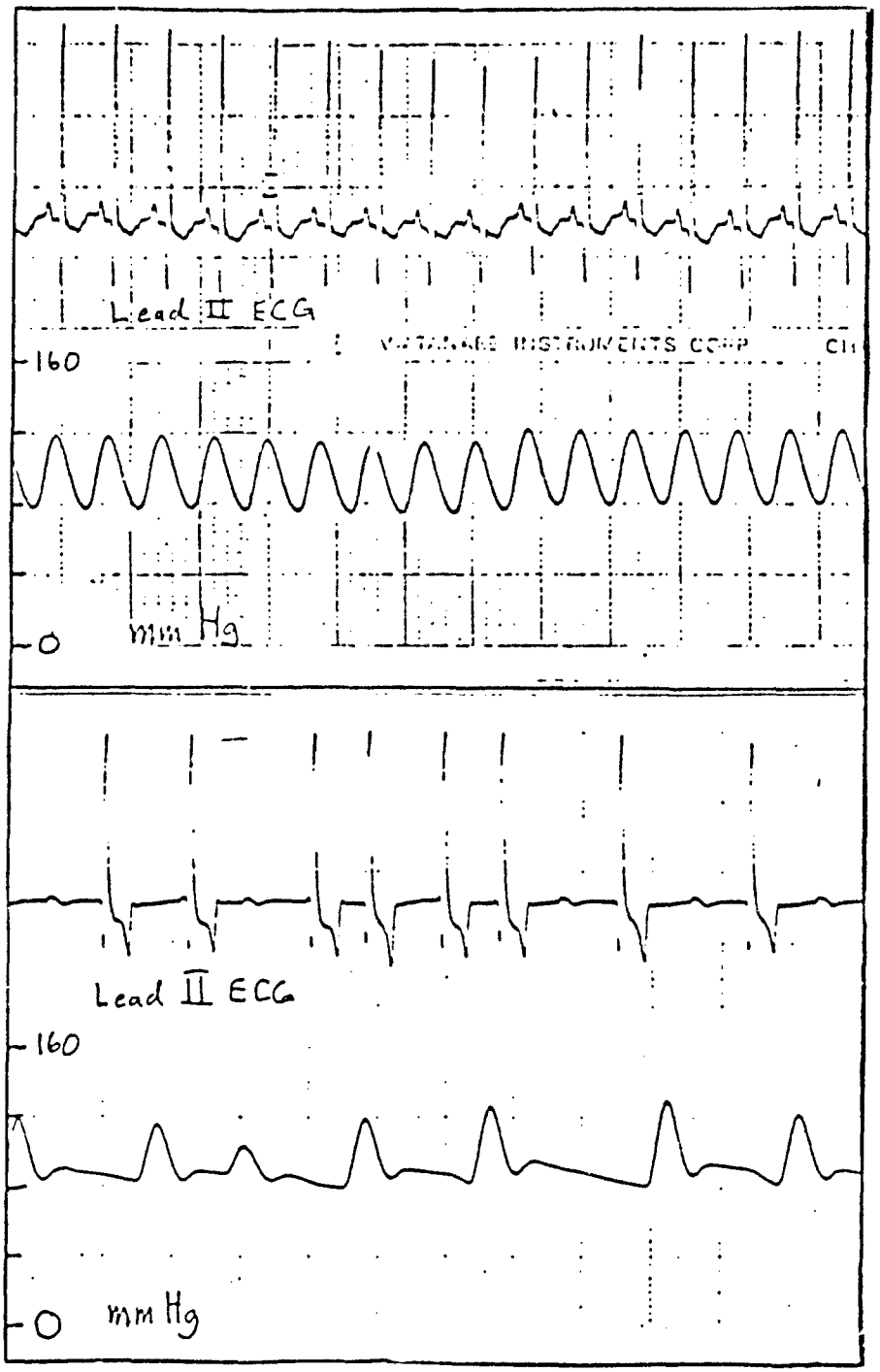


Figure 5. These two records (from the same experiment as Figure 4) show how roridin-A prolonged the PR interval (80 msec to 320 msec.) and markedly increased T wave amplitude (negative in the canine lead II ECG). Upper panel was before, and lower panel was 2 hr. after roridin-A was injected.

Significance

Intravenous T-2 and roridin-A elevate sinus rate (tachycardia) by activating release of catecholamines (directly or reflexly). After exposure to these toxins for more than 1 hour (3 mg./Kg.), pacemaker arrest was observed (either sinus arrest or sino-atrial block).

PROJECT II. ELECTROPHYSIOLOGIC ABNORMALITIES PRODUCED BY TRICHOTHECENES IN ISOLATED HEARTS

Table 1 shows the significant changes in isolated atrial activity that took place after 20 min. of perfusion of 4 molar T-2 toxin. Sinus rate fell from 222 to 142 beats per min. Action potential duration at 90% repolarization decreased from 55 to 21 msec. And the interval between activation of right atria and right ventricles increased from 48 to 70 msec. After 30 min. perfusion (or with higher toxin concentrations) disturbances in rhythm and conduction were observed.

Each Polaroid print in Figure 6 contains right atrial action potentials above and right ventricular electrograms below. The control record is Panel A. After 20 min. of 4 moles/L. toxin perfusion, sinus rate was slower and transient periods of ventricular tachycardia were observed (Panel B). Panel C shows that whenever atrioventricular conduction did occur, the A-V interval was prolonged. Panel D shows the record after 30 min. of toxin perfusion. Atrial and ventricular tachycardia were present as was complete A-V block.

To further confirm this atrioventricular dissociation, a right atrial and a right ventricular cell were simultaneously impaled; there was no correspondence between atrial and ventricular action potentials.

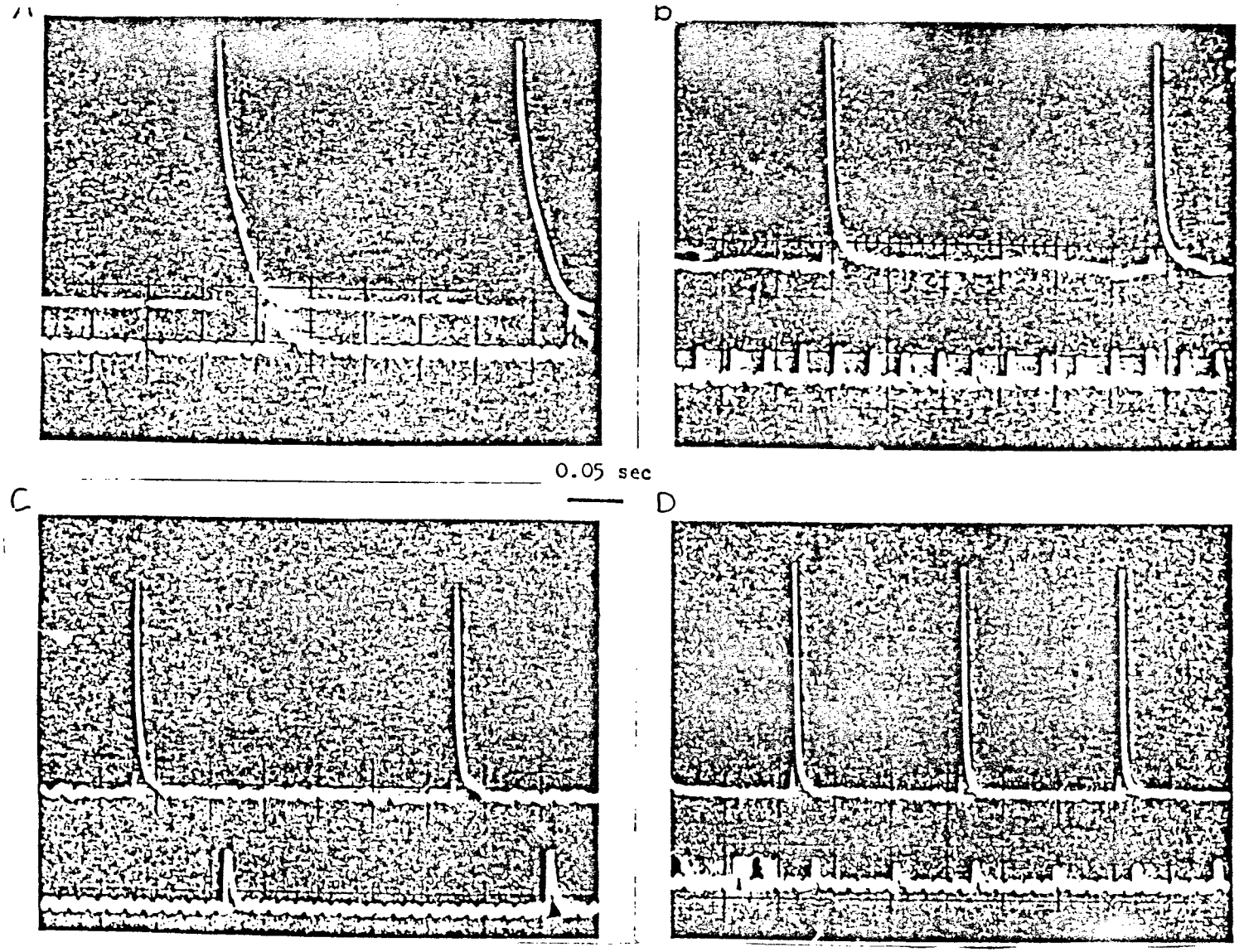


Figure 6. Right atrial action potentials and right ventricular electrograms shows response to 4 micromolar T-2 toxin at 20 min. (B and C) and 30 min. (D). Details discussed in text.

Changes in sinus rate, atrioventricular conduction, and action potential morphology observed in this study can be caused by releaase of endogenous acetylcholine. To test this possibility, atropine (5 mg./L.) was added to the perfusate to block the acetylcholine receptor. After such treatment and exposure to T-2 toxin for 30 min., there was no slowing of sinus rate and no shortening of the action potential.

Figure 7 shows the response to 30 min. perfusion of 10X higher concentration of T-2 toxin for 20 min. The upper print shows a slow atrial firing rate, A-V block, and ventricular quiescence. 10 min. later the lower print shows long periods of atrial quiescence interrupted by brief periods of atrial tachycardia.

In summary,

1. All trichothecenes tested up to 1 ppm or 40 micromoles/L. caused atrial, ventricular, and A-V conduction disturbances.
2. Automaticity and A-V conduction were extremely sensitive to the trichothecenes.
3. Some changes were prevented by atropine, but not A-V block.
4. Effects could be reversed quickly by washout with toxin-free solution.

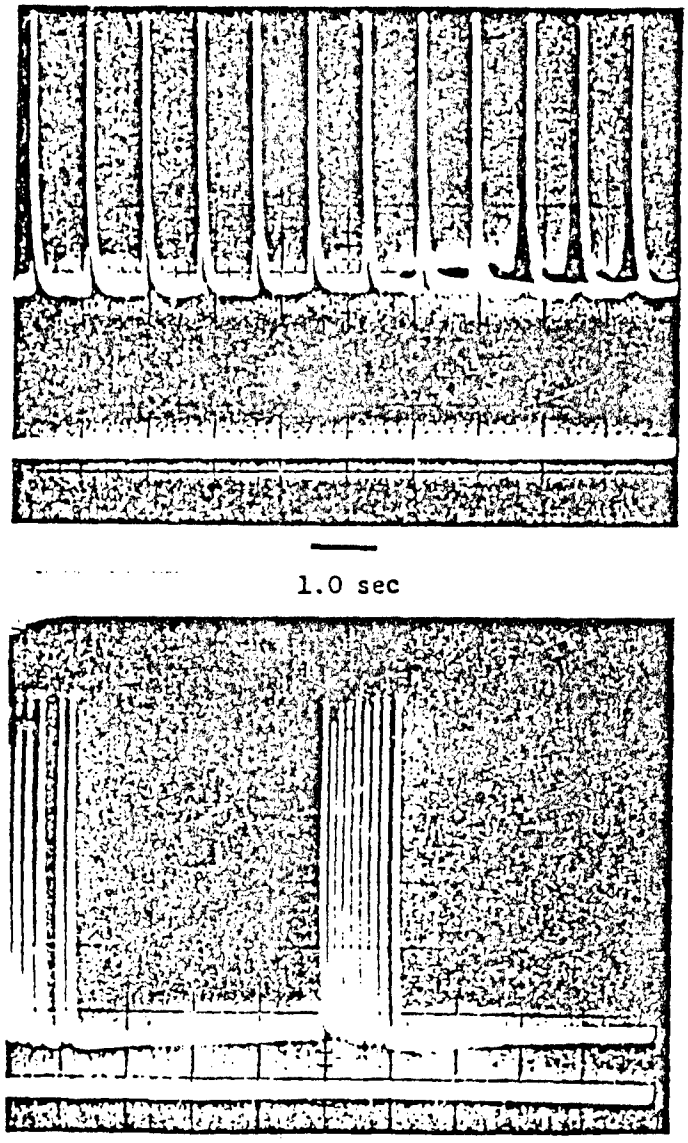


Figure 7. Recordings identical to those in Figure 6. Details are discussed in text.

PROJECT III. TRICHOTHECENE-INDUCED ACTION POTENTIAL CHANGES IN CANINE ATRIAL WORKING MYOCARDIUM.

To review findings in canine ventricular working muscle cells, Tables 2 and 3 summarize the eight quantitative action potential parameters that were measured before and after exposure to 1 mg./L. mycotoxin (control).

The action potentials from ventricular (papillary) muscle cells shown in Figure 8 illustrate the typical effects of trichothecene mycotoxins in canine ventricular cells. T-2 tetraol, for example, reduced the total duration of the ventricular cell action potentials from 320 ms. to 245 ms. (Figure 4B), and lowered the plateau (arrow) from 14 mv. to 4 mv.

Papillary muscle cell action potentials were significantly shortened by the T-2 ($p < 0.05$), but the CV and MDP remained unchanged. The overshoot was reduced from 23 to 17 mv. ($p < 0.05$) and the total amplitude was reduced correspondingly. Sixty minutes exposure to 1 mg./L. T-2 produced no significant changes in the action potential parameters of ventricular (septal and free-wall) muscle cells.

Table 4 summarizes the effects of scirpentriol. Ventricular muscle cell action potentials were significantly altered by scirpentriol. The action potential duration was shortened ($p < 0.05$), resting potentials, were depolarized by 11.5 mv. ($p < 0.05$), and the total amplitude was reduced

by approximately the same amount ($p < 0.05$). It is interesting to note that scirpentriol had no effect in the false tendon cells.

Table 5 summarizes the effects of T-2 tetraol on ventricular muscle cell action potentials. T-2 tetraol depolarized papillary muscle cells by 16.5 mv. ($p < 0.05$), which was reflected in the reduction of the total amplitude ($p < 0.05$), and also reduced dV/DTmax by 50% ($p < 0.05$). In ventricular muscle T-2 tetraol reduced the action potential duration ($p < 0.05$), but no other parameters were altered.

Table 5 shows that T-2 shortened the action potential duration in papillary muscle cells ($p < 0.05$), and similarly, scirpentriol shortened the action potential duration of ventricular muscle cells ($p < 0.05$).

ATP counteracted the effect of T-2 on the papillary muscle cell action potential duration and it also counteracted the shortening effect of scirpentriol on the ventricular muscle cell action potential duration.

In light of the action potential changes observed in ventricular cells, potential effects on atrial working cells were explored. Right atria were isolated from 20 separate hearts and arterially perfused with a physiological solution. Addition of T-2 toxin (30 mg./L.) increased atrial cell resting transmembrane potential by 10 ± 7 mv. Addition of roridin-A (30 mg./L.) increased resting potential by 9 ± 5 mv. In contrast to observations in ventricular cells, no changes in action potential duration were observed in canine atrial working muscle cells.

Table 2. Control action potential parameters for 3 ventricular cell types

	AMPLITUDE (mv)	OVERSHOOT (mv)	dV/dTmax (V/S)	COND.VEL (M/S)	MDP (mv)	APD (20) (ms)	APD (50) (ms)	APD (80) (ms)
FALSE TENDON								
X	119.9	31.2	258.5	0.953	87.8	40.8	150.8	208.5
sd	9.2	5.5	45.3	0.325	8.1	8.6	37.5	34.3
(n)	(13)	(13)	(13)	(10)	(13)	(13)	(13)	(13)
PAPILLARY MUSCLE								
X	98.1	22.9	151.3	0.190	75.5	105.3	190.3	230.0
sd	8.6	3.1	39.5	0.040	10.2	33.4	46.1	55.8
(n)	(15)	(15)	(12)	(5)	(15)	(15)	(15)	(15)
VENTRICULAR MUSCLE								
X	98.2	21.0	127.1	0.178	77.5	107.1	206.5	246.2
sd	10.3	4.4	35.8	0.126	8.0	23.6	25.3	26.0
(n)	(17)	(17)	(14)	(8)	(17)	(17)	(17)	(17)

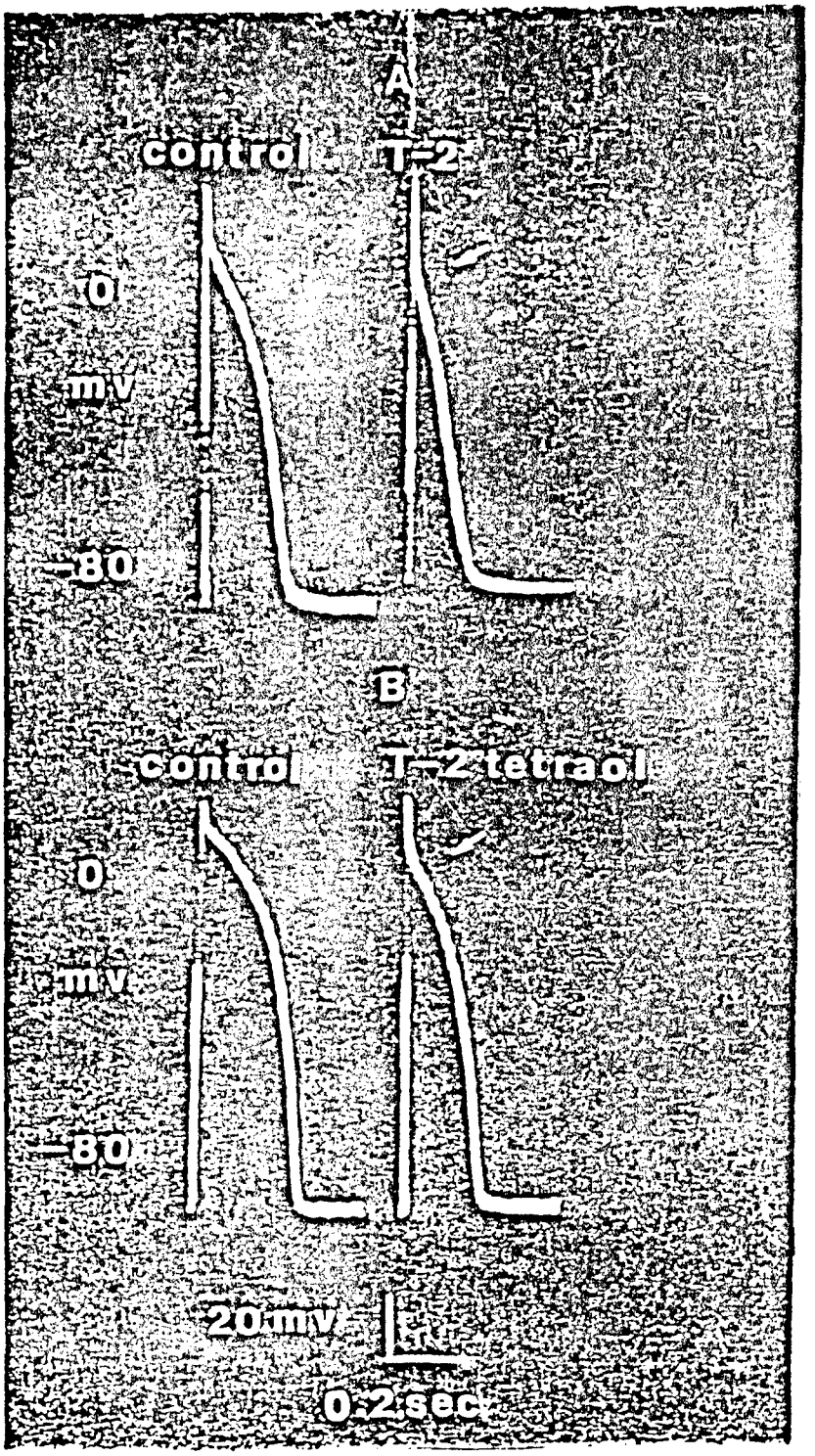


Figure 8. Action potentials from canine false tendon cells (A), and canine papillary muscle cells (B) before and after 60 minutes exposure to trichothecene mycotoxins (1 part per million).

Table 3. The effect of T-2 toxin on the action potential parameters of 3 ventricular cell types.

	AMPLITUDE (mv)	OVERSHOOT (mv)	dV/dTmax (v/s)	COND.VEL. (M/s)	MDP (mv)	APD(20) (ms)	(APD(50) (ms)	APD(80) (ms)
FALSE TENDON								
X	98.3	32.1	214.3	0.538	74.4	27.9	82.9	136.4
± sd	14.6	7.2	99.3	0.178	6.7	7.6	9.9	22.1
(n)	(7)	(7)	(7)	(4)	(7)	(7)	(7)	(7)
sig.	*	ns	ns	*	*	**	**	***
PAPILLARY MUSCLE								
X	87.9	17.0	139.3	0.219	79.6	49.9	111.4	134.3
± sd	7.9	4.4	19.2	0.055	5.0	16.1	11.4	9.3
(n)	(7)	(7)	(7)	(4)	(7)	(7)	(7)	(7)
sig.	*	**	ns	ns	ns	***	***	***
VENTRICULAR MUSCLE								
X	91.7	19.3	123.3	0.095	75.8	117.5	192.5	225.0
± sd	6.1	3.9	29.3	0.004	3.1	30.3	27.2	23.5
(n)	(6)	(6)	(6)	(4)	(6)	(6)	(6)	(6)
sig.	ns	ns	ns	ns	ns	ns	ns	ns

* = $p < 0.05$, ns = $p < 0.05$ comparisons are with the control for each tissue. (see Table 1), MDP = maximum diastolic potential, dV/dT max. = maximum rate of rise of the upstroke, ADP = action potential duration

Table 4. The effects of scirpentriol on the action potential parameters of 3 ventricular cell types

	AMPLITUDE (mv)	OVERSHOOT (mv)	dV/dTmax (v/s)	COND.VEL. (M/s)	MDP (mv)	APD(20) (ms)	(APD(50) (ms)	APD(80) (ms)
FALSE TENDON								
X	120.7	32.0	230.0	1.120	88.7	40.0	115.0	171.7
± sd	3.1	2.0	17.3	0.453	2.3	0.0	0.0	7.6
(n)	(3)	(3)	(3)	(2)	(3)	(3)	(3)	(3)
sig.	ns	ns	ns	ns	ns	ns	ns	ns
PAPILLARY MUSCLE								
X	97.5	26.5	115.0	——	70.5	121.2	222.5	263.8
± sd	6.0	3.0	13.2	——	6.8	23.6	17.1	16.0
(n)	(4)	(4)	(4)	——	(4)	(4)	(4)	(4)
sig.	ns	*	ns	——	ns	ns	ns	ns
VENTRICULAR MUSCLE								
X	82.3	19.4	160.0	——	66.0	75.0	151.7	203.3
± sd	28.6	8.0	20.0	——	18.4	37.2	55.7	44.5
(n)	(6)	(6)	(3)	——	(6)	(6)	(6)	(6)
sig.	*	ns	ns	——	*	*	**	**

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Comparisons are with the control for each tissue (see Table 1). MDP = maximum diastolic potential, dV/dT max. = maximum rate of rise of the upstroke, ADP = action potential duration.

Table 5. The effects of T-2 tetrool on the action potential parameters of 3 ventricular cell types.

	AMPLITUDE (mv)	OVERSHOOT (mv)	dV/dTmax (v/s)	COND.VEL. (M/s)	MDP (mv)	APD(20) (ms)	(APD(50) (ms)	APD(80) (ms)
FALSE TENDON								
X	123.7	28.3	291.7	0.533	95.3	40.0	191.6	251.7
± sd	12.7	3.5	12.6	0.412	12.9	0.0	46.5	37.5
(n)	(3)	(3)	(3)	(3)	(3)	(3)	(3)	(3)
sig.	ns	ns	ns	ns	ns	ns	ns	ns
PAPILLARY MUSCLE								
X	78.0	19.0	75.0	——	59.0	125.0	230.0	270.0
± sd	0.0	4.2	21.2	——	2.8	21.2	42.4	42.4
(n)	(2)	(2)	(2)	——	(2)	(2)	(2)	(2)
sig.	*	ns	*	——	*	ns	ns	ns
VENTRICULAR MUSCLE								
X	98.2	21.3	125.0	0.171	71.5	51.3	137.5	180.0
± sd	20.6	10.4	57.4	0.023	11.1	16.5	29.0	23.1
(n)	(4)	(4)	(4)	(4)	(4)	(4)	(4)	(4)
sig.	ns	ns	ns	ns	ns	***	***	***

*** = $p < 0.001$, MDP = maximum diastolic potential, APD = action potential duration
** = $p < 0.01$
* = $p < 0.05$

Significance

The nature of changes produced by T-2, roridin-A, and other trichothecenes in cardiac working muscle cells (atrial and ventricular) do not suggest that they are likely substrates for trichothecene-induced arrhythmias. Thus, the conduction sysstem of the atrium (sinus node pacemaker and A-V node) appears to be a likely candidate.

PROJECT IV. TRICHOTHECENE-INDUCED ACTION POTENTIAL CHANGES IN CANINE FALSE TENDONS.

Table 2 summarizes the eight quantitative action potential parameters that were measured for each tissue prior to exposure to 1 mg/L mycotoxin (control). These were compared to the same action potential measurements taken after exposure to the trichothecene mycotoxins.

The action potentials from false tendon cells and ventricular muscle cells (shown in Figure 8) illustrate the typical effects of trichothecene mycotoxins in canine cardiac cells. T-2 caused the false tendon cells to depolarize 10 mv. The total duration was reduced from 245 ms to 185 ms, and the plateau voltage (arrow) was reduced from 16 mv to 6 mv. T-2 tetraol reduced the total duration of the ventricular cell action potentials from 320 ms to 245 ms, and lowered the plateau (arrow) from 14 mv to 4 mv.

Table 6 summarizes the effects of T-2 mycotoxin on the action potential parameters of false tendon cells and papillary muscle cells. T-2 reduced the total amplitude of false tendon cell action potentials by 21 mv ($p < 0.05$), this reduction was due to depolarization since the overshoot was not significantly altered by T-2. The conduction velocity in false tendons slowed significantly ($p < 0.05$), and APD20, APD50, and APD80 were reduced ($p < 0.05$). The dV/dTmax. remained unchanged by exposure to T-2.

Papillary muscle cell action potentials were significantly shortened by the toxin ($p < 0.05$), but the CV and MDP remained unchanged. The overshoot was reduced from 23 to 17 mv ($p < 0.05$) and the total amplitude was reduced correspondingly. Sixty minutes exposure to 1 mg/L T-2 produced no significant changes in the action potential parameters of ventricular muscle cells.

Table 4 summarizes the effects of scirpentriol on the action potentials of canine false tendon cells, papillary muscle cells, and ventricular muscle cells. The R2, R3 hydroxylated metabolite had no significant effect on the action potential parameters of canine false tendon cells or papillary muscle cells. Ventricular muscle cell action potentials however were significantly altered by scirpentriol. The action potential duration was shortened ($p < 0.05$), the cells were depolarized by 11.5 mv ($p < 0.05$) and the total amplitude was reduced by approximately the same amount ($p < 0.05$). T-2 altered these parameters in the false tendon cell tendon cell action potentials, and had no effect on the ventricular muscle cells. Scirpentriol had no effect on the false tendon cells but significantly altered ventricular muscle cell action potentials.

Table 5 summarizes the effects of T-2 tetraol on canine false tendon cells, papillary muscle cell and ventricular muscle action potentials. The hydroxylated metabolite had no effect on the false tendon cell action potential parameters. T-2 tetraol depolarized papillary muscle cells by 10.5 mv ($p < 0.05$), which was reflected in the reduction of the total amplitude ($p < 0.05$), and also reduced dV/dTmax by 50% ($p < 0.05$). In ventricular muscle T-2 tetraol reduced the action potential duration ($p < 0.05$), but no other parameters were altered.

Table 3 shows that T-2 shortened the action potential duration in papillary muscle cells ($p < 0.05$), and

similarly, scirpentriol shortened the action potential duration of ventricular muscle cells ($p < 0.05$). The addition of adenosine to the suffusate had no effect on the shortened action potentials. ATP (2 mM/L) produced no changes in action potential parameters of papillary muscle cells or ventricular muscle cells from the controls. However ATP counteracted the effect of T-2 on the papillary muscle cell action potential duration and it also counteracted the shortening effect of scirpentriol on the ventricular muscle cell action potential durations.

PROJECT V. ELECTROPHYSIOLOGIC EFFECTS OF TRICHOTHECENES ON CANINE SINUS NODE PACEMAKER CELLS

Table 6 summarizes changes in sinus node and A-V conduction observed in the isolated rat heart. Right atria were isolated from 20 separate hearts and arterially perfused with a physiological solution. Addition of T-2 toxin (30 mg.L.) decreased the sinus rate by 17 ± 11%. Addition of roridin-A (30 mg.L.) slowed the sinus rate by 15 ± 7.5%. Sino-atrial (SA) block was observed in half of the preparations perfused with roridin-A and was eliminated when roridin-A was removed. Maximum diastolic potential of sinus node pacemaker cell, became hyperpolarized by 6 ± 3 during exposure to roridin-A (30 mg./L.). Therefore, direct effects on automaticity were progressively developing.

Significance

Although the predominant responses to intravenous T-2 and roridin-A are hypotension and reflex tachycardia, simultaneous direct effects on sinus node pacemaker cells develop progressively. Sinus node firing rate becomes slower as pacemaker cell maximum diastolic potential becomes more negative, and this can progress to sino-atrial block or sinus arrest. If at the same time the A-V junction substitute pacemaker were to become suppressed, this would be a potentially lethal electrophysiologic event. Mechanisms for pacemaker suppression are being investigated by techniques described in this report. A rationale for

reversal of these effects may emerge based on this information.

PROJECT VI. MECHANISMS OF ACTION OF TRICHOTHECENES AT THE CELL MEMBRANE LEVEL.

Cardiac Cell Dispersion and Culture. Houser and associates (33) modified some existing techniques for dispersion of adult cat hearts into individual cells. This technique applies equally well to the other mammalian hearts in which it has been tested, including the dog heart. We have recently adapted the dispersion technique to our isolated, perfused canine atrial preparation (Figure 9), and we find that a high yield of atrial cells can be reproducibly harvested from hearts of any age (Figures 10 and 11). Critical points in this procedure appear to be 45 min. arterial perfusion with 0.1% collagenase (Sigma Chem. type 5), $[Ca^{++}]$ = 0.03 millimole/L of less, and maintenance of normal arterial pressure and temperature.

The collagenase-treated tissue is minced with scissors and mildly agitated in 10 mL. Ca^{++}-free perfusate for 10 min. It is then filtered through a nylon mesh (200 micron pore diameter) and bovine serus albumin (Sigma Chem.) is added (final concentration = 1%). For long-term culture experiments sterile technique is practiced. All solutions are passed through a 0.2 micron filter before contacting tissue. All tools are sterilized by autoclave. Final steps in the dispersion are carried out in a laminar flow hood (NuAire 300) to maintain sterility. After the mincing step the tissue fragments are mildly agitated in 10 mL. Ham's

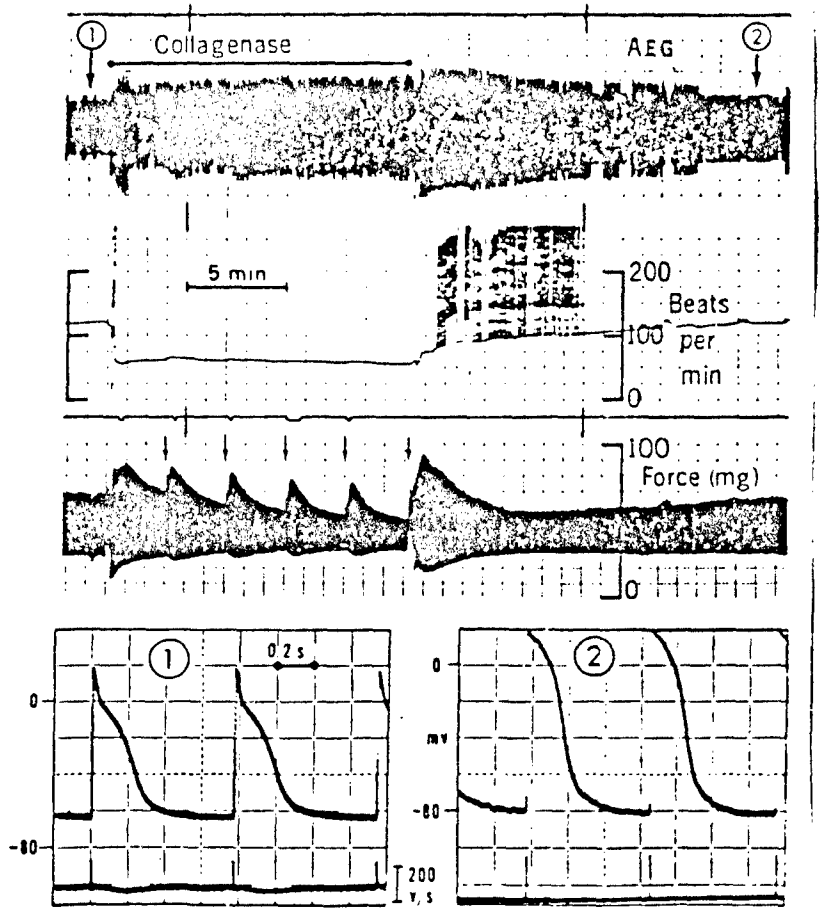


Figure 9. Evidence is provided that collagenase does not damage electrical function in the isolated canine atrium. After 15 min. exposure to 0.1% collagenase, atrial electrophysiology recovered completely. (From Woods, J. Molec. Cell. Cardiol. 16:843-850, 1984).

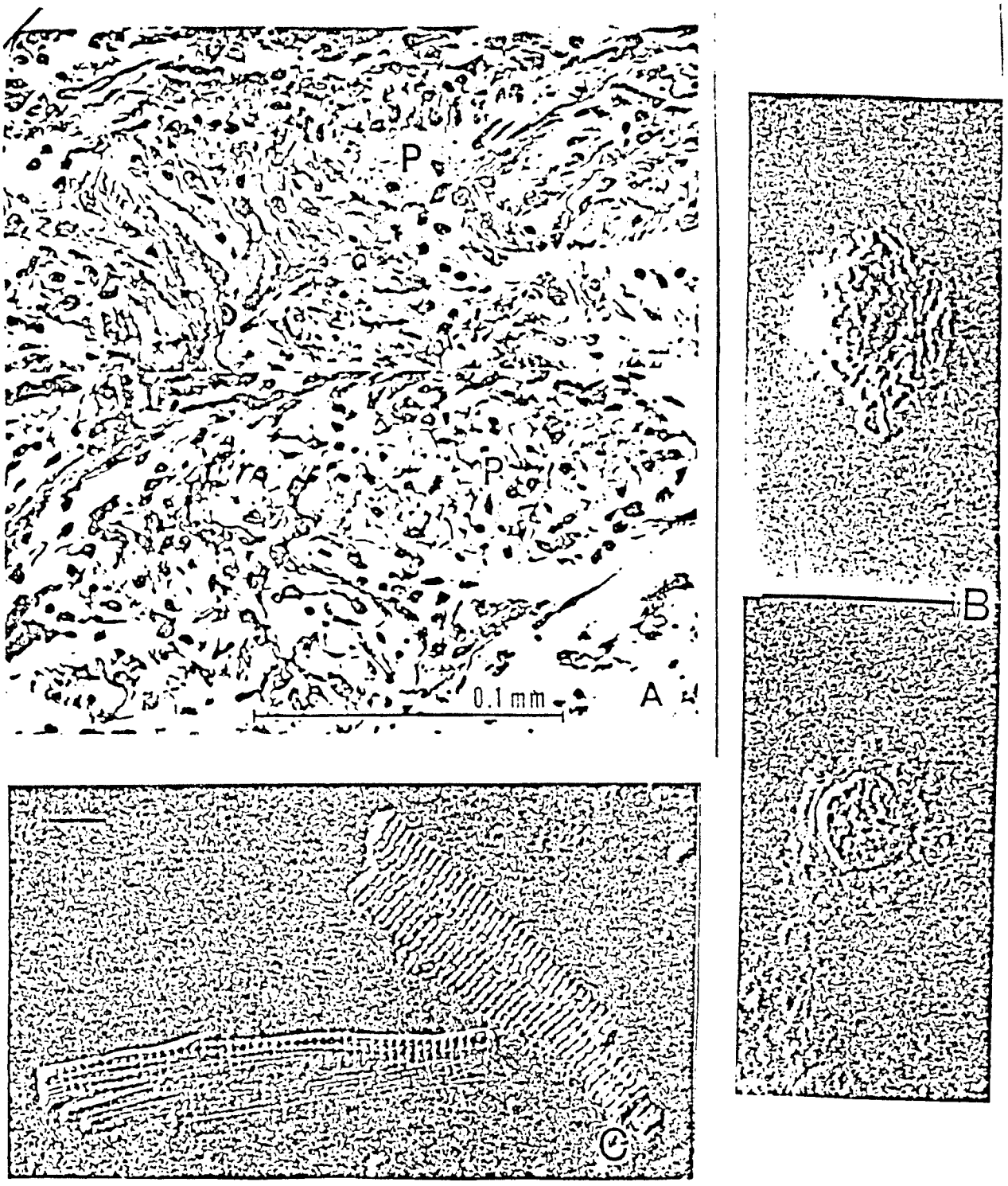


Figure 10. A. Examples of isolated nominal pacemaker cells (B) and atrial working cells (C) in culture. For comparison one of a series of whole sinus node sections (A) from a canine heart is shown in A (from Woods et al., Circ. Res. 39:76-82, 1976). Cells were photographed through a Nikon Diaphot microscope. Calibration bars are 20 microns.

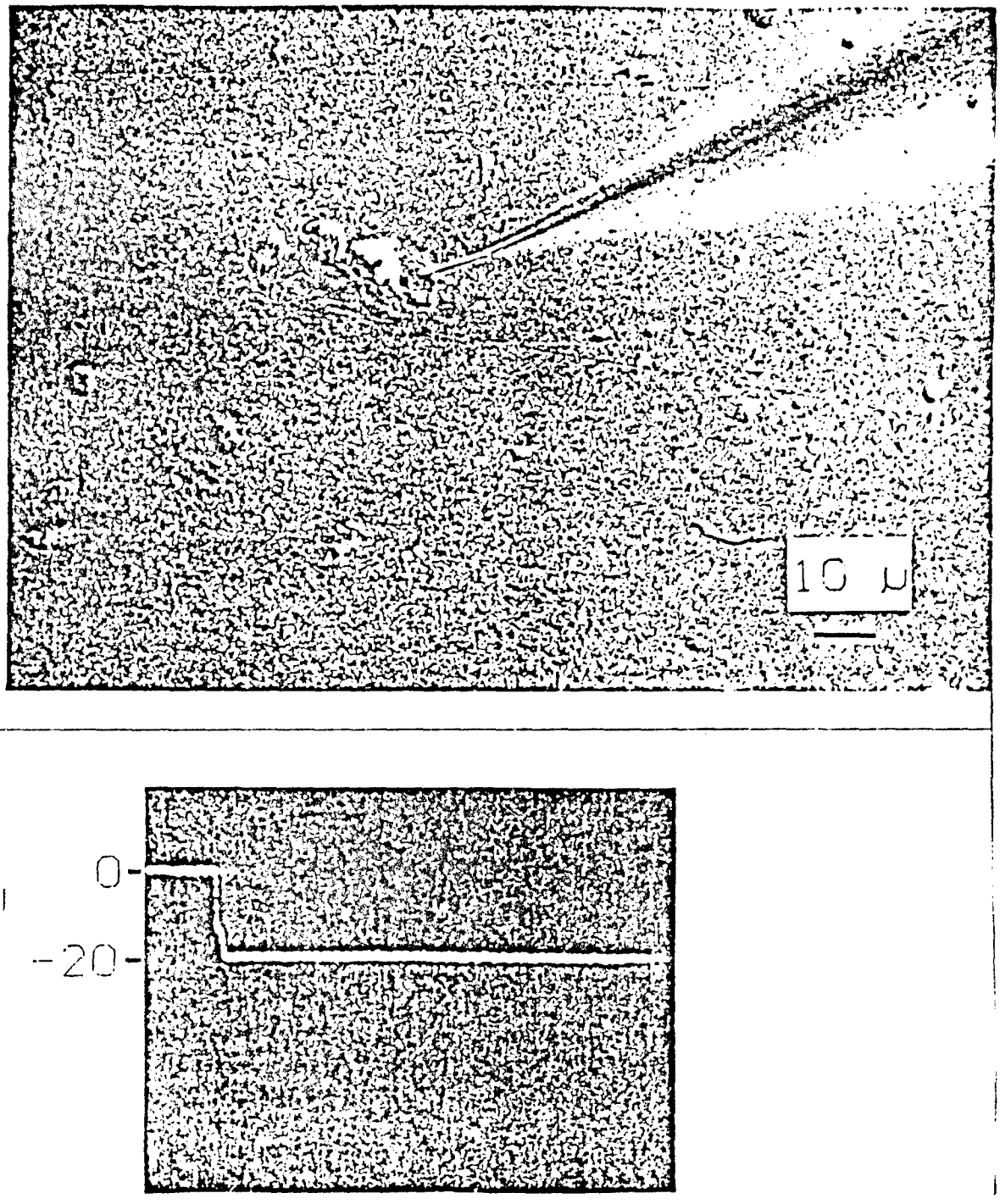


Figure 11. Isolated cluster of nominal pacemaker cells. One cell is impaled by a microelectrode. Record of transmembrane potential typically recorded at moment of impalement is included.

F-12 Dulbecoo's Modified Eagle's Medium (DME) in a 1/1 ration with 1% dialyzed fetal bovine serum and 50 units/mL. each of penicillin and streptomycin (Irvine). The medium also contains 0.2 mmol./L. L-glutamine. Tissue debris is strained with sterile nylon mesh and DME is replenished to bring the final volume to 10 mL. Aliquots (2.0 mL.) are transferred to 3-cm. diameter Falcon culture dishes (5 per atrium or sinus node).

The dishes are stored in a water-jacketed, humidified 5% CO_2 incubator (Forma 3158) at 37°C. Aliquots (0.2 mL.) are aseptically removed from them and placed in a glass-bottomed chamber (0.5 mL.). In this chamber cells adhere to the poly-lysine treated bottom so that they can be impaled and so that they can be suffused with fresh media. Cells are viewed through an inverted microscope (Nikon Diaphot) resting upon a compressed gas suspension table (Micro-G) to suppress vibration. Cell density is typically 50 ± 25 cells per field of view at 400 power magnification.

Electrophysiology: Patch Clamp. Suitable constructed microelectrodes can remove a patch of cell membrane and record the passage of current through it (34). Both transmembrane potential and chemical composition of the solutions bathing the patch will be controlled to determine what kinds of ion-channels are present (details below). Micropipettes (from 1.0 mm. outside diameter borosilicated glass) will be pulled in a 2-stage process (with a modified Stoelting puller) so that the final product will have a 2.0 micron outside diameter tip with 0.5 micron diameter opening. It will again be heated in a microforge to remove any jagged edges. The shaft of the pipette will be insulated with a layer of Sylgard all the way to the tip. The opening will be abutted against a cell membrane.

Negative pressure in the pipette lumen will draw the membrane tightly against the opening and the membrane will

bond electrostatically to the exposed glass ring of the pipette tip. When the membrane bonds to the entire ring of glass, it creates a high resistance (giga-ohms). Thus, current passing through the pipette traverses the relatively lower resistance of the membrane patch. This current can be both injected and recorded with the Axopatch system on order.

The membrane patch can be left attached to the intact cell membrane or it can be removed, which will be desirable when the ionic composition of the solution exposed to the (formerly) intracellular membrane surface must be controlled precisely. Trans-patch voltage gradient will be set with the List EPC7 patch clamp amplifier. At certain levels of transmembrane potential voltage-sensitive ion-selective channels open and close rapidly and these appear as high frequency (depending on temperature and voltage) current spikes of constant amplitude (3 picoamperes or multiples of three in Figure 4). The amplitude of the current depends upon the trans-patch potential in the manner shown in Figures 12, 13 and 14. Current carriers can be identified by changing the concentrations of Na^+, K^+, Ca^{++}, etc. to alter the driving forces on them during the channels' open phases.

Acceptable patches will have the following characteristics: 1) at least 10 ohms resistance; 2) a signal-to-noise ratio greater than five; 3) stable recording for sufficient length of time to permit experimental

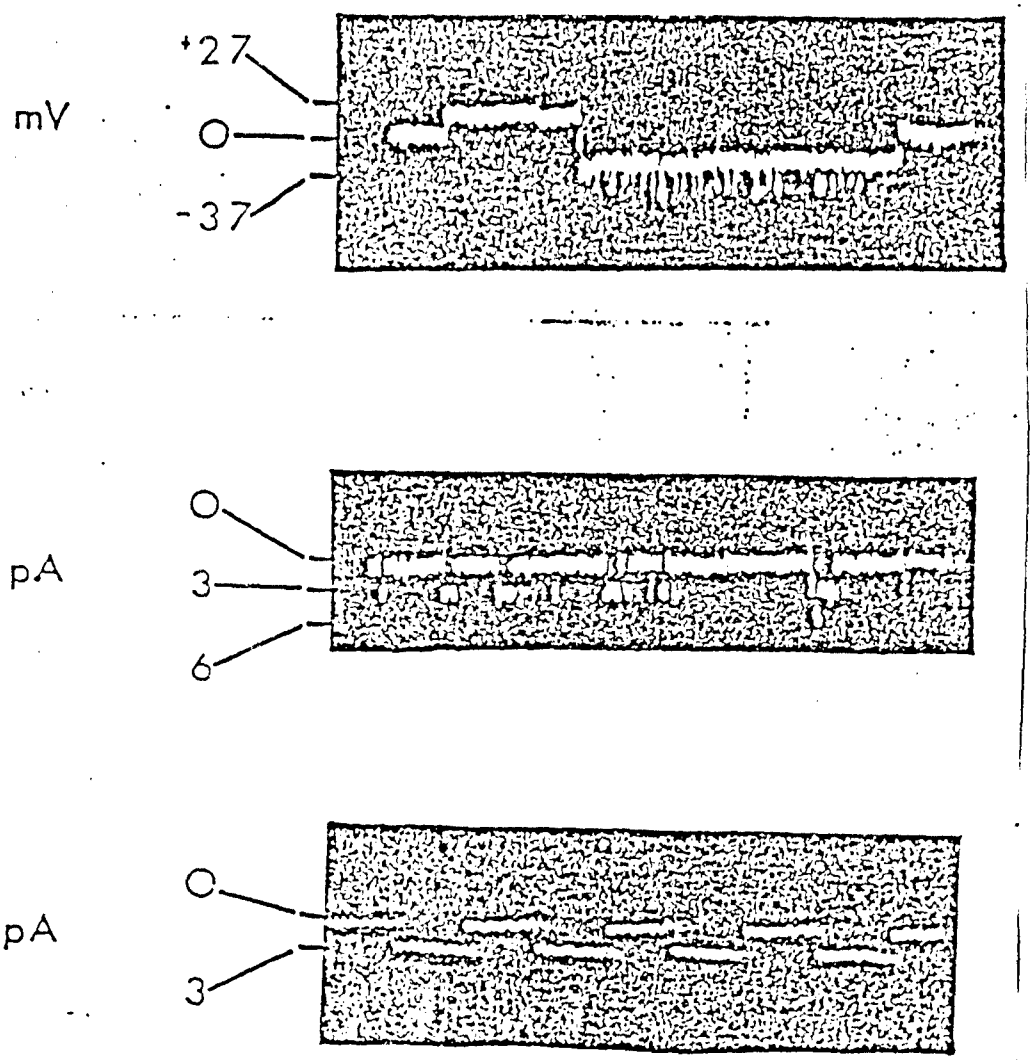


Figure 12. Amplitudes of currents recorded in a rat ventricular myocyte during channel openings at holding potentials of -37, 0, and +27 mv.

procedures; and 4) with detached patches, no vesicle formed on the pipette tip. A rise time of the channel opening of less than 100 sec will indicate that a single membrane is on the tip, i.e., no vesicle. A vesicle on the tip would be indicated by an increase in the capacitance of the channel current.

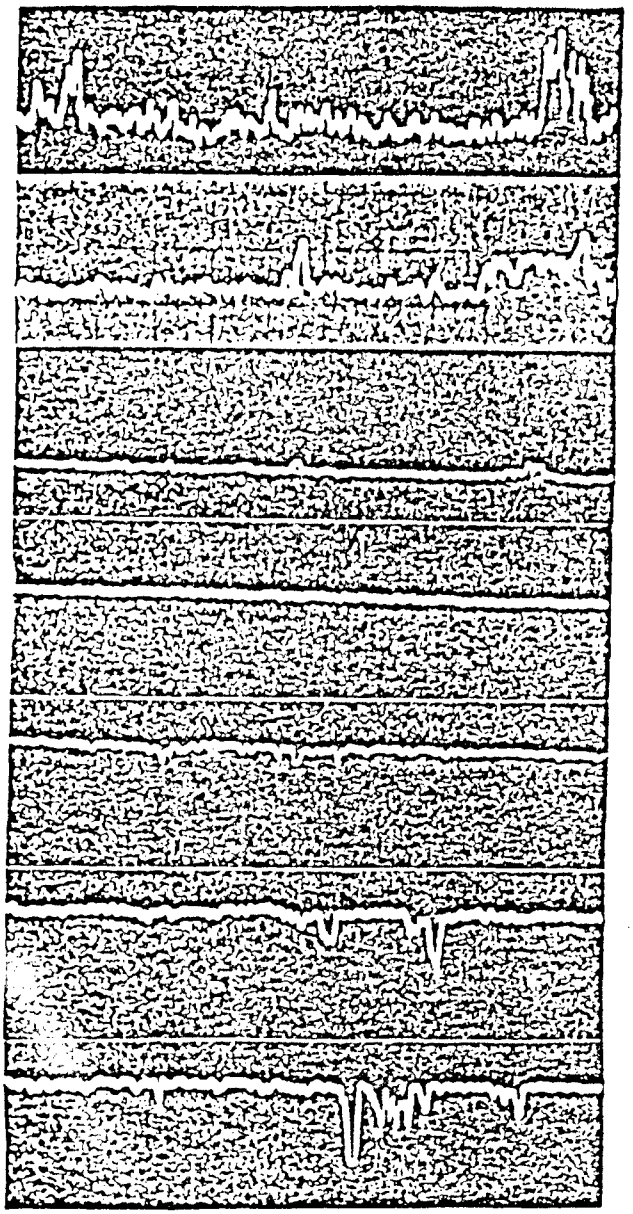


Figure 13. Current traces recorded from a patch of dog atrial working muscle cell membrane during voltage steps to (top to bottom) -60, +40, +20, 0 (resting potential), -20, -40, and -60 mv (electrode potentials). Electrode solution contained (in millimoles/L) 150 Na^+, 2.0 Ca^{++}, 154 Cl^-, 5 HEPES, pH = 7.25; External solution = 150 Na^+, 2.0 Ca^{++}, 5 HEPES, pH = 7.4. Seal resistance = 1 Gohm; cell was attached. Inward current is downward.

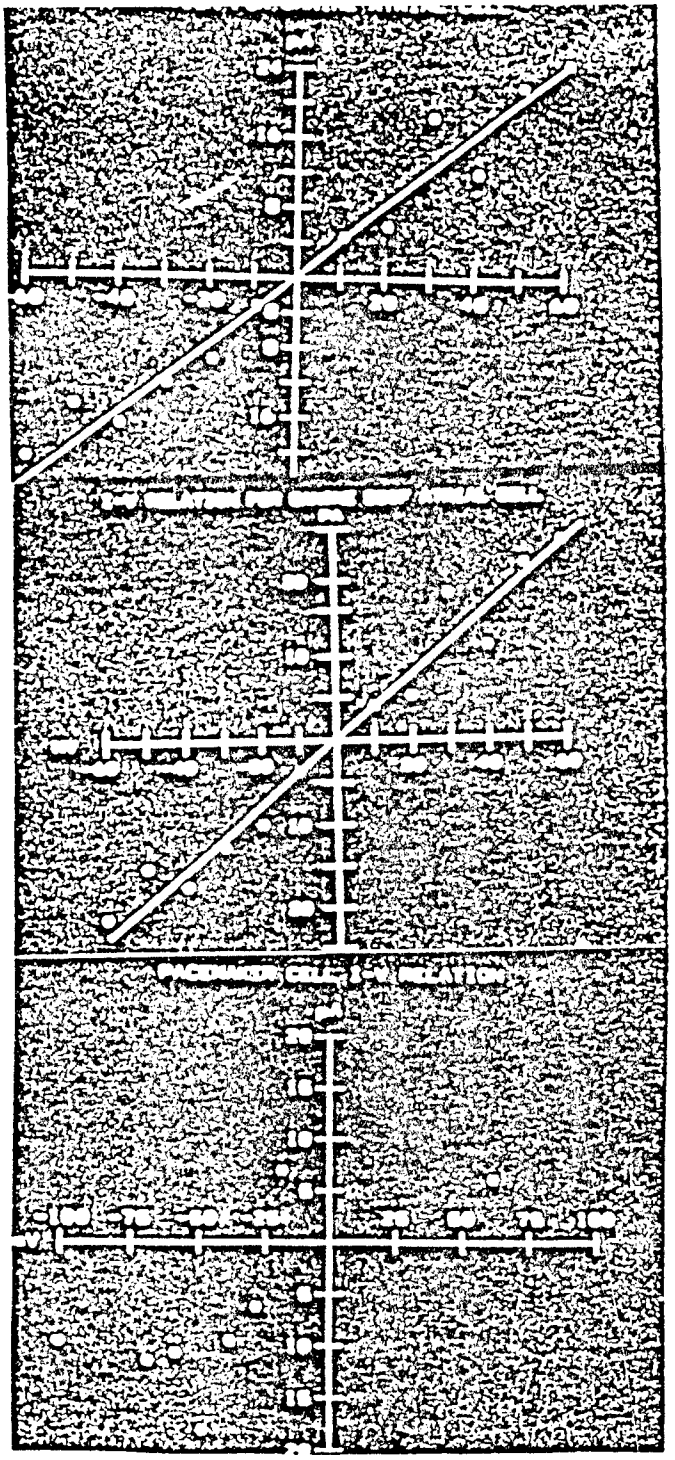


Figure 14. Current/voltage plots for a canine atrial working muscle cell after 1 day in culture (upper) and after 10 weeks in culture (middle). Data from one single patch on a nominal pacemaker cell gave points shown in the bottom plot.

To investigate characteristics of K^+ channels, K^+ will be the only cation present and an impermeant anion will be used (such as gluconate). Thus, KOH will be titrated to pH 7.4 with gluconic acid. For inside-out or cell-attached type patches, the bath will contain 150 mM. K gluconate, 5 mM. HEPES, and the pipette will contain the same plus 1 mM. Ca^{++}. With inside-out patches and the same K^+ concentration on both sides, the reversal potential is zero, i.e., no current seen at mv. In other inside-out or cell-attached type patches, the reversal potential will be determined. In these cases various combinations of bath and pipette solution compositions (75 mM., 150 mM., or 300 mM. K gluconate) will result in varying reversal potentials depending upon the direction of K^+ concentration gradient. (Channel activity in membrane patches does not appear to be affected by change in solution osmolarity in our experience.)

To detect the presence of Ca^{++}-activated K^+ channels, the Ca^{++} concentration in the pipette of inside-out patches will be varied (in different patches) between 1 and 2 mM. The Ca^{++} concentration in the bath of outside-out patches will be varied also between 0 and 2 mM. The Ca^{++} concentrations less than 1.0 mM. will be buffered to insure accurate concentrations by using appropriate combinations of Ca^{++} and EDTA (Handbook of Physics and Chemistry).

To detect Na^+ channels, NaOH will be titrated with gluconic acid, and 75, 150, and 300 mM. Na gluconate, 5 mM.

HEPES, pH 7.4, solutions will be used as described above. For Cl^- channels, 75, 150, and 300 mM. NaCl, 5 mM. HEPES, pH 7.4 will be used similarly. CsCl will be used instead of NaCl to rule out effects of Na^+ per se.

To detect Ca^{++} channels are studies Ca gluconate will be prepared from CaOH and gluconic acid. Combinations of Ca^{++} concentrations ion the pipette and bath will be varied between 0 and 10 mM.; Cs gluconate will make up the remainder of the ionic concentration. All of these Ca^{++} solutions will contain 5 mmolar HEPES. All solutions will be filtered (0.2 micron) and bubbled with 95% O_2 - 5% CO_2, pH 7.4 in the storage reservoir prior to filling pipette or chamber.

The specificity of the channel for an ion will be determined by the reversal potential predicted by the Nernst equation (when only one permeant ion is present), or by the Goldman-Hodgkin-Katz equation (when multiple premeant ions are present).

After the proper configuration of the patch is obtained, the patch membrane will be voltage-clamped at levels between ±100 mv. in 20 mv. increments for seconds. As the channels open and close the currents necessary to maintain the constant voltage are recorded. When Na^+ channels are to be studied, voltage pulses of approximately 100 msec. will be used, because the Na^+ channels inactivate with time. To observe channel activity at reduced frequency some experiments will be conducted at 12°C.

For qualitative data analysis, we will observe the data (played back from the magnetic tape) on the screen of the oscilloscope and recorded on the chart recorder. Channel characteristics such as single opening, bursts, flickering, subconductance states and amplitude can easily be assessed in this manner. For quantitative analysis we will use a computer program (software developed and obtained from Dr. Henry Lester). The analysis requires an IBM PC with a Techmar Labmaster analog interface board (Cleveland, Ohio). The software system is written in BASIC; subroutines will be used to capture and analyze single-channel events in an off-line mode. 32 sec. will be required to analyze each channel opening. The program will also perform the following analyses: 1) estimate the baseline; 2) detect channel opening and closing; 3) verify that an opening or closing occurred; and 4) record channel voltage sensitivity (amplitude as a function of applied voltage) to estimate conductance, open time, closed time, and frequency of occurrence.

After mapping the isolated, arterially perfused sinus node with microelectrodes to locate precisely the region of interest, we will disperse the sinus node cells with collagenase (0.1%, described below). After the dispersed cells are transferred to culture dishes (5.0 mL.), individual cells and clusters of cells will be observed in an inverted microscope. The nominal pacemaker cells and working muscle cells will be visually identified and impaled

with electrodes to record a spontaneous electrical activity (studies in this laboratory and others to date have revealed only quiescence). The technique used to disperse nominal pacemaker cells reduces the working muscle cell population of the sinus node sample. Therefore, a simultaneous dispersion of right atrial working muscle cells from the right atrial appendage will be carried out. Aliquots (2.0 mL.) from each population will be combined in separate culture dishes. The culture of mixed cell types will be observed at intervals of 6 hours thereafter to determine when 2 different cell types unite and when spontaneous electrical or mechanical activity begins. Pilot studies have indicated that such events occur after 48 ± 12 hours in mixed cell culture. After 12 ± 2 days the working muscle cells lose their characteristic appearance, so the experiments will terminate at this time. If modification of cell culture conditions leads to longer retention of original cell structure, the durations of experiments will be extended.

Another series of experiments will use micromanipulation of 2 different cell types (nominal pacemaker and working muscle) to bring their cell membranes into apposition with each other. The objective will be to find out how spontaneous formation of cell couplings takes place in cutlure. We will be testing the possibility that connexons form whenever the 2 cell membranes become tightly apposed and we will determine how long it takes them to

develop. In these experiments transmembrane potentials will be recorded as monitors of intercellular communication.

The yield of nominal pacemaker cells and other types in the typical dispersion is greater than 5,000, so 1 dog per week will be sufficient to satisfy the needs of all projects requiring nominal pacemaker cells in culture.

Two separated nominal pacemaker cells will be impaled individually. They will be micromanipulated into contact with each other. Current pulses will be injected into one cell, and if any current passes through the other cell membrane, the other microelectrode will record a proportional change in potential. When the membranes are first apposed, intercellular resistance is high, so little current, if any, passes into the second cell. When a low resistance path is created between the 2 cells, they begin to communicate electronically. More current becomes diverted through the second cell. We will detect this with the microelectrode in the second cell.

(6) Discussion and conclusions

Each completed project let to a conclusion that is listed below and discussed subsequently.

I. Systemic trichothecenes (T-2 and roridin-A) elicit circulatory catecholamines that accelerate heart rate. However, prolonged exposure (1 hour or more) was associated with cardiac pacemaker arrest.

II. T-2 toxin and roridin-A each disrupt cardiac impulse conduction directly by depressing conduction system

activity; this disruption reverses when toxins are removed.

III. Working muscle cells (atrial and ventricular) are not affected significantly by trichothecenes.

IV. Trichothecene effects are arrhythmogenic in the ventricular conduction system (fals tendon cells) and can be reversed by perfusing adenosine triphosphate.

V. Direct effects on pacemaker (sinus node) and conductory cells (atrioventricular node) can be best assessed mechanistically by studying membrane channels.

VI. These cells can be studied in primary cell culture. Their single channel conductance properties could reveal the direct actions of trichothecenes (continuing work on this project).

This list of conclusions points toward the next step in this research program. It establishes that T-2 toxin and roridin-A both attack the cardiovascular system in 2 very specific ways. One is by causing release of neurotransmitters from autonomic nerves. The other is by selectively inhibiting transmembrane current in the cardiac conduction system. This program will continue to focus on how trichothecenes interact with nerve and heart cell membranes to exert their potentially lethal effects.

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ABSTRACTS

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